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One message on the state of science

The new Presidential Science Advisor last week gave the House of Representatives a vision of how smaller budgets may bring strength. He was partly disingenuous; may he also be right?

The United States Congress has a built-in flair for the theatrical. So much is clear from the circumstances that brought Dr George Keyworth and Dr Frank Press (his predecessor as Presidential Science Advisor now transmogrified to president of the National Academy of Sciences) to the same witness table last week (see page 601). True to expectation and perhaps even to form, Dr Keyworth, the government representative, talked confidently of how his government's policies would make the United States research enterprise more and not less efficient. Dr Press, a little on the defensive, offered a compact between the research community and the United States Administration that would assure basic research in the United States of modestly rising budgets and the United States government of the cooperation of the research community in transferring funds from the 'less productive areas or institutes'. Both witnesses were talking about the future, naturally enough. Neither of the set speeches mentioned the 12 per cent cut in federal budgets which President Reagan declared in August but which Congress will probably, in the end, decline to give him. But Dr Keyworth's vision was of a future so distant that, by the time it arrives, he may again be an ordinary person not bound to support the policies of the administration of the day.

Whatever the time scale of their dreams, however, Keyworth and Press agreed last week on one essential point — that the federal government has a continuing responsibility for the support of basic research. Keyworth argued that the private sector cannot be expected to support a sufficient effort in basic research, given its short-term need for profit; Press tended to quote President Lyndon Johnson on the subject. One obvious difficulty, which each of them might have mentioned, is that Congress is unlikely to endorse such a fine distinction between basic research — the pursuit of generally unpopular academics — and what is called applied research and demonstration. Why should a congressman, never more than two years from his reelection ordeal, vote for the general distribution of research funds to the generality of academics when he could vote instead for his own state's favourite federal laboratory or demonstration project? Dr Keyworth should have been prepared to remind Congress of its own responsibilities. Dr Press would no doubt have been prepared to back him up with accounts, gathered at first hand, of how the pork barrel rolls.

For the immediate future, the outstanding question is whether the scientific community is in a position to do what Dr Keyworth asks of it, and to use the system of peer-review committees, and of the advisory committees that advise the grant-making and mission-oriented agencies respectively, in such a way as to concentrate funds for basic research in fields of enquiry and on institutions that are likely to make best use of them. Broadly speaking, the answer is that it all depends. For Press is also right to imply in what he said last week that the research community can do this kind of job when it believes that it can do so safely, knowing that its paymasters will not take its recommendations of places or people from which funds can be withheld as licences to go ahead while saying that recommendations of where support should be increased can wait until the economy has improved. Dr Keyworth's speech last week, full though it was of references to a creative basic research community x years from now, conspicuously lacked a sense of responsibility towards — or even regret for — those who have contributed to the same endeavour in

the past x years. Keyworth was looking not at the future but at the horizon beyond it.

Press's recipe for the immediate future is, unfortunately, equally unpalatable. Faced with present economic uncertainties, the United States government could not agree to a compact that would assure basic research of some specified rate of growth each year. The economic crisis that afflicts science in the United States (and elsewhere) has arisen precisely because people are uncertain about the economic future. Other institutions are similarly afflicted. None can be insulated. Keyworth last week would have been wrong to promise that they might be. The best course for him and Dr Press for the weeks ahead will be to make plain that nobody is owed a living, but that the government will do what it can, within the resources at its disposal, to make the best use of what can be spent on basic research.

Two minor scandals stand out from last week's exchange. Dr Keyworth argued in his statement that things may not be too bad because the Pentagon's spending on basic research will be increasing in the years ahead. One trouble is that what the Department of Defense counts as basic research is not always what other people understand by the term. Another is that the Pentagon's sense of what is excellent is often compromised by its sense of what may be expedient. If the traditional and trusted sources of support for basic research are to be cut back but the Pentagon enlarged and the research community as a result given less of a say in what is excellent and worthwhile, should not Dr Keyworth have been urging that this year's increase in the Pentagon's operating budget should be matched by a corresponding increase of the budgets of the agencies dedicated to (and skilled at) providing support for basic research?

The second scandal that will stick in people's minds is Dr Keyworth's homily last week about the neglect of science and mathematics in American high schools. Since the post-sputnik era in the late 1950s, enthusiasm for the physical sciences has evidently waned. Rightly, Dr Keyworth shares with other observers a sense of anxiety about the consequences. Will United States universities in future be providing remedial mathematics as freely as they now have to offer remedial English? Or will it simply be that the brightest young people follow different kinds of courses, letting American industry depend on skilled people recruited from abroad? The fears may be exaggerated, but Dr Keyworth's defence of his government's inaction is equally an exaggeration of the doctrine that Republican governments are ideologically opposed to intervention in other people's affairs. Keyworth said that the Administration is concerned at the decline in high school studies in the physical sciences, but that education is a matter for state and local government and that he had confidence that, sooner or later, they would see the light. Can he be sure, especially when the government for which he works has decided (as it did in its March budget) that the National Science Foundation should be effectively robbed of funds for the further development of the science curriculum? If the benefits of basic research are too distant to be left to industry, can it safely be assumed that the long-term needs of the educational system can be entrusted to embattled school committees? And if not, does not the Administration have a duty to show the way? The National Science Foundation's educational programme may have aged ungracefully, but the Administration cannot wash its hands of the set of problems its science adviser has identified.



Finniston's pale shadow

The British Engineering Council now exists. Its first task is to define for itself a role.

Engineering education promises to be in the 1980s a lightning-rod for public solicitude to take the place occupied in the 1950s by the cause of motherhood. In the United States last week, Dr George Keyworth was wringing his hands before Congress in anxiety about the difficulties of recruiting and then keeping members of university engineering faculties — but was confident that the market would somehow find a way. In Britain, engineering education has been a public issue for at least four years, since Sir Monty Finniston's committee was set up. Broadly speaking, there have been three distinct complaints. First, there is not enough engineering education. Second, what there is is either inappropriate or not good enough or both. And third, in universities as in the world outside, engineering has a lower status than science. Finniston's report (now nearly two years old) embraced all these views and more, and urged that there should be an Engineering Council equipped with powers over engineering curricula in all kinds of teaching institutions and with the funds with which to implement reforms. Implicitly, Finniston asked that his council should supersede the existing engineering institutions, some of them monuments to the Industrial Revolution.

The contrast between the United States and Britain is instructive. In both countries, it is acknowledged that engineering skill is one of the mainsprings of industrial innovation, productivity and prosperity. There, however, the similarity ends. Dr Keyworth last week was merely echoing the longstanding complaint that American industry is so fully seized of the economic importance of engineering skill that universities cannot easily compete for teachers — and graduates flock off to lucrative jobs in industry undiverted by the prestige of the academic life. In Britain, much the same may be happening in fields of engineering linked with computer engineering, but for the most part those carrying a torch for British engineering are still mouthing hackneyed slogans — that engineering (and engineers) should be accorded more "status" by everybody in sight.

This is the spirit in which the Committee of Engineering Professors has in the past few months been urging that the Science and Engineering Research Council should be split into two, one part concerned with the support of engineering research in universities. For, the argument seems to be, how can engineers respect themselves without a research council to call their own? (The proposal, mercifully, seems to have been headed off by the willingness of what used to be the Science Research Council to mention engineering in its title.) Similarly, there is excited talk, from people such as Lord Caldecote, the president of the Engineering Fellowship (another pressure group), that the new council may help to give the engineering professions public esteem. What these complainants consistently overlook is that the exercises in public relations on which they are embarked are merely palliatives, and that British engineering would be better off if its esteem were reflected in the eagerness of British industry to snap up young engineers — and to pay them decent salaries.

How is that to be accomplished? And will the new council help? Much will depend on its willingness to come to grips with the educational questions which Finniston grasped too firmly — like a man picking up a stinging nettle. In spite of the inquiries of the past four years, nobody is much wiser about the balance that should be struck between the various ingredients in various kinds of engineering courses. Instead, at the university level, the assumption is being established that there should be two kinds of courses, one lasting three years and one for four.

Unfortunately, very little thought has been given to the academic content of these courses, and the suspicion remains that university engineering courses, like those in other educational institutions, are too narrow because the separate engineering institutions too jealously insist that courses should concentrate on the topics judged to be relevant to their fields of interest.

Mechanical engineers worry about the design of gear wheels, civil engineers about the design of bridges, electrical engineers about the design of circuits and so on. The influence of the engineering institutions stems from their willingness to grant exemption from some of the requirements for professional qualification to students who have followed approved courses. This right will remain under the new arrangements. But who is to say that these are the lines on which engineers should be taught? Might not a preparation in something called engineering science be a better preparation for work in a profession that must turn its hand to almost everything? And how is the engineering profession to come to terms with the disconcerting truth that many whose academic training is quite different from that now required of engineers still function in industry as engineers? It would be splendid if the new council chose, as its constitution allows, to license the occasional physicist or chemist to practise as an engineer. Better still, it should take a fresh look at what academic preparation is needed by those who may in future keep British industry alive. But it should firmly acknowledge that a demand for more prestige for engineers is tantamount to a demand that water should run uphill.

Who pays what piper?

British universities and football clubs are in trouble. Can they make common cause?

Economic crisis is at least even-handed. Last week, British newspapers were full of gloomy prophecies of how no fewer than a dozen among three-score British institutions are threatened with bankruptcy in the months ahead. The causes are easily described. Running costs have risen. The cost of employing staffs has become doubly onerous — the annual salary bill is high, but the longer-term implications of people's contracts with their institutions are a shadow over the future. But income is falling. Traditional customers can no longer afford to take advantage of the services the institutions exist to provide. Inevitably, where the threatened institutions command particular loyalty from the cities in which they are based, especially when they are the only one of their kind, bands of loyal supporters have formed to conjure a phoenix from the impending ashes. Elsewhere, newspaper columnists comfortably bemoan the passing of a revered tradition and a past source of public pleasure and enlightenment. The institutions are the professional (commercial) football clubs that play with a round ball, otherwise known as soccer or "association football" clubs.

British academics reading this torrent of gush (which may be made meaningless if only the clubs can negotiate a better television contract next season) may be forgiven for not recognizing that much of the purple prose could apply to the institutions for which they work. The proportions at risk are about the same. The inexorable increase of expenditure has similar causes. And income is falling because the government, effectively the only customer, cannot afford to pay for the services on offer. But may not academics, most of whose institutions will be making critical decisions about their future this week and next, learn from what the football clubs are planning?

Many football clubs plan to make better use of their fixed plant; universities might similarly offer courses out of business hours or in vacations. Other clubs plan to increase their ancillary income; selling franchises to put university coats of arms on tee-shirts would hardly be lucrative, but what of the benefits from academic enterprises with which British universities have so far only flirted? Some clubs plan to sell off players, presumably the most saleable and thus probably the best; but universities are thinking of offering early retirement only to the least employable. No football club lacks a local constituency, a group of people to which is has given pleasure in the past and which is now prepared to fight that it should have a future; too many universities, unfortunately, are unable to make a comparable claim on their localities. In a macabre way, it will be interesting to see which threatened group does best in the months ahead.

Keyworth and Press on basic science

Researchers to decide where funds go?

Washington

In the first coherent statement of the Reagan Administration's thinking on science policy, Dr George (Jay) Keyworth, the president's science adviser, said last week that the scientific community must be prepared not only to help identify areas in need of increased support, but also to say where support should be decreased.

The time of constantly growing budgets across the board had passed, Dr Keyworth said, and the scientific community's "best and most thoughtful judgement" was needed to identify areas to deemphasize as well as those meriting new emphasis. "To those who object to such undertakings, and to all of my scientific colleagues, I must say that if scientists do not make such choices, others will, but with less acuity."

Dr Keyworth was addressing a hearing of the House of Representatives Science and Technology Committee on the implications for science of the present budget reductions. At several points in his testimony, Dr Keyworth claimed that the Reagan Administration was promoting a fundamentally new approach to the government's responsibilities for science and technology. "I am proposing a federal role in research and development which is appropriate to the 1980s — appropriate to a national mood which calls for increased vigour and acceptance of responsibility by individuals and organizations in the private sector and decreased involvement by the federal government in many of our affairs", Dr Keyworth said.

Yet apart from promoting the need to trim research budgets to fit straitened economic circumstances, much of the substance of what Dr Keyworth had to say was similar to statements to the committee made by his two predecessors, Dr Frank Press, science adviser to President Carter and Dr Guyford Stever, adviser to President Ford. All three stressed the federal government's responsibility to promote basic research as a long-term investment in the future. And all agreed that short-term research and development should primarily be the responsibility of the private sector.

Dr Keyworth, in line with the overall policy of the Reagan Administration, emphasized that science policy had to be carried out in the context of other national policies such as national security, international relations, energy, social services and the economy. "For example, science policy, made without considering econo-

mic policy, is irrelevant", he said.

Dr Keyworth's immediate predecessor, Dr Frank Press, now president of the National Academy of Sciences, while accepting the need for financial restraint, gave last week's committee hearing a sharply different view. He advocated a ten-year "compact" between government, industry and universities to establish new national goals for the support of science. This would include commitment to an annual real growth of between 1 and 2 per cent in the scientific research budget; an additional 1 per cent growth to support special "targets of opportunity" in different fields; and a real growth of 1 per cent, equivalent to about \$50 million a year in the contribution made by industry to university research.

To a large extent, however, the differences in the statements from Dr Keyworth and Dr Press were a reflection more of their different constituencies than of disagreements on substance. Both agreed that there should be a reduction in federal support for "demonstration projects" that should properly be taken over by the private sector. And Dr Press spoke of Dr Keyworth's "courage" in

making uncomfortable decisions about what research projects should be cut.

Responding to a question about press reports that he had recommended the elimination of all new planetary exploration missions by the National Aeronautics and Space Administration (NASA) over the next decade — a proposal being discussed this week by President Reagan and NASA administrator James Beggs — Dr Keyworth emphasized the many scientific gains to be made from space shuttle missions, and suggested that this was one area in which direct scientific comparisons might be made.

Expanding on a theme of several of his earlier speeches, Dr Keyworth told the committee that there were several reasons why the United States could not expect to be preeminent in all scientific fields. He also warned against the tendency to avoid tough decisions about which projects should be cut by applying cuts uniformly across all fields. "I believe the discipline of making such hard choices will ultimately benefit science, just as the occasional pruning of a tree can promote, rather than retard, its heal."

David Dickson

Spain has the money but not the people

Science in Spain seems to be embarked on a period of rather heady growth. Money under the direct control of the Spanish science research council, the Consejo Superior de Investigaciones Científicas (CSIC), will triple next year if decisions taken by the lower house of the Spanish parliament are confirmed by Senate. This comes on top of a doubling last year of the centrally administered "funds for research", from which CSIC draws the largest single portion. One sign of how the wind is blowing is the story of a Spanish chemist, recently returned from the United States, in a poorly-equipped CSIC laboratory. In the past few weeks he has found himself spending money "eight or nine hours a day" bringing his laboratory up to scratch; and he has not even had to submit a formal grant application.

CSIC plays a major role in science in Spain, maintaining 23 institutes in the humanities and 77 in natural sciences and employing 1,600 scientists plus 4,400 supporting staff. But the Consejo is not the only beneficiary of increased spending. Parliament has also agreed to create two new research funds, on which universities (and others) will be able to draw: one to be controlled by the Ministry of Industry, and one by the Ministry of Health, together increasing the government research budget by about a quarter.

The regional distribution of support is also becoming a shade more equitable. Whereas some 85 per cent of the central funds for research have usually been spent

in Madrid, parliament this year has insisted that 25 per cent be spent in the regions; and the autonomous government of Catalonia is also expected to increase its fledgling research budget next year, from 100 million pesetas (around £500,000) to 200–250 million pesetas.

In total, over the past two years Spanish spending on research and development has nearly doubled as a fraction of gross national product from around 0.3 per cent to 0.5 per cent now — still way below the developed world average of something over 2 per cent but an increase which is already proving difficult to manage.

The problem is people. The truly effective Spanish research community is very small, and there is a government freeze on new appointments. This is having two serious effects, which some feel must be corrected quickly or the new money will be merely wasted.

First, there are not enough highly qualified scientists to act as critical referees in all fields in which Spain would wish to do research; although arrangements have been made for refereeing all proposals, in practice the judging has proved to be imperfect and partisan. An international refereeing system is proposed by those who would survive it; the others — inevitably the majority — resist.

Second, there are too few posts becoming vacant to employ the rising tide of experienced Spanish scientists who wish to return from other countries and make use of the new money. So paradoxically the coffers are open, but there are good

scientists unemployed.

The man who may change all this is the new minister for universities and research, Professor Federico Major, recently returned from Paris after a spell as Deputy Director-General of UNESCO. Major, a 47-year-old developmental neurobiologist, has kept in touch with science despite a long career in politics. He is a personal friend of King Juan Carlos, and yet is right wing (he was minister of universities under Franco) and so may be able to ride the strong conservative opposition in Spain. He has to steer through a new law for the universities (now entering its seventh draft). And he appears to have been impressed, during his time in Paris, by the new French commitment to science as a means of economic development. He is already talking of a "law for science" which would define a budget and a programme for a stretch of a few years, so clearing all political hurdles in one jump. This is exactly the strategy of the French minister for science. It will be interesting to watch how far Professor Major will mimic him.

Robert Walgate

UK biotechnology

Blood money

A British publicly-funded organization for encouraging innovation in industry has made its second major investment in the fledgling biotechnology industry. The British Technology Group (BTG — an amalgamation of the former National Research Development Corporation and the National Enterprise Board) announced this week that it is investing £2 million in Speywood Laboratories Limited of Nottingham for the development of new techniques for blood protein manufacture. Prutec Limited, a subsidiary of the Prudential Assurance Company, will be matching BTG's investment.

BTG's other biotechnology venture is Celltech, the company it established last year with three finance houses and the Medical Research Council to develop recombinant DNA techniques for manufacturing medical products, including monoclonal antibodies. The group's latest venture is to develop a new fractionation technique for manufacturing blood proteins, an area in which Celltech is not involved.

Speywood, a small company set up seven years ago, will use the £4 million roughly to double the size of its factory and research laboratories. Its initial aim is to improve its polyelectrolyte process for fractionating blood cryoprecipitates, which allows the separation of a greater variety of blood proteins than the traditional Cohn process for fractionating whole-blood plasma. The company has already used the new technique for isolating a pure form of factor VIII from pigs' blood for use in particularly sensitive human patients. Clinical trials are expected next year. It now plans to expand factor VIII

production and extend the technique to factor IX, factor VWF (for treatment of von Willebrand's disease) and fibronectin.

Speywood is the only commercial company now producing blood products in Britain, the bulk of production being controlled by the health department through its Blood Products Laboratory at Elstree, and Speywood's plans for expansion are likely to be welcomed by the National Health Service, which has to import a large proportion of its supplies.

Speywood's longer-term goal, however, is to develop and use recombinant DNA techniques for manufacturing blood proteins such as factors VIII and IX, fibronectin, α_1 antitrypsin and albumin.

David Heath, managing director of Speywood, welcomes investment from BTG and Prutec not only for the money but also because it gives the company access to expertise on recombinant DNA technologies in universities (BTG acts as a "broker" between industry and the universities). He hopes that the £4 million will launch the company into an expansion plan costing about £19 million over the next five years. Later on, he will be looking for further investment, but BTG is for the time being non-committal, preferring to see how the company shapes up before committing itself further. **Judy Redfearn**

Telecommunications

French hanging on

Brussels

French doubts about the wisdom of opening up some public purchasing contracts in telecommunications to its partners in the European Economic Community held up agreement last Monday on a set of recommendations designed to stimulate the growth of the European market in telecommunications.

Although the recommendations would not be binding, they constitute a first step towards achieving community-wide services and a community-wide market for terminal and other kinds of equipment. The telecommunications administrations of the various member countries have, of course, been cooperating for a long time but the supply of equipment for national networks has tended to remain in national hands. Following the meeting of the Council of Telecommunications Ministers in December 1977, the European Commission established a working group on future networks which has recommended urgent action in the field of digital networks. But the different policies being pursued and conflicting commercial considerations continue to hamper progress.

The first recommendation is for consultation with a view to ensuring that new services are introduced within the community only when they are mutually compatible. Second, the telecommunications administrations of member

Pleasant surprise

The shortfall in admissions of overseas students to British universities has not been as great as anticipated. According to the University Grants Committee (UGC) admissions of overseas undergraduates in 1981–82 totalled 4,918 compared with 5,017 in 1980–81, a shortfall of about 2 per cent. Post-graduate admissions were 7,414, only 0.5 per cent less than in 1980–81.

These figures, however, come on top of reductions in 1980–81 compared with 1979–80. The sharpest reductions then were in students from the United States and Malaysia which sent 1,105 and 3,988 undergraduates respectively, compared with 1,450 and 4,188 in 1979–80. Most other countries cut back by two to five per cent. The exceptions were West Germany, Hong Kong and Nigeria which sent more students than in 1979–80. The largest contributors of overseas students are still Malaysia, Hong Kong and the United States, between them sending more than half of Britain's total overseas enrolments.

As yet UGC has no figures for individual universities or for the countries of origin of new entrants for 1981–82, but the unexpectedly small decline should mean that some universities, at least, have not suffered as severe a loss of income from overseas students' fees as had been feared. The effect may be to lessen the impact of the government's cut in the university grant. **Judy Redfearn**

countries should not discriminate between domestic and other EEC suppliers of telematic terminal equipment by means of type approval procedures taking longer than six months, or which are more complex and costly to comply with than those employed elsewhere. But it was the third recommendation which caused the greatest problems. This specified that for the period 1981–83, telecommunications agencies should seek tenders from suppliers who manufacture in other community countries for at least 10 per cent of their annual orders.

The French quibbled about the phrasing "suppliers who manufacture", partly because, as one diplomat put it, "they don't want to go ahead with the recommendations anyway". But the French also want to avoid loopholes that will open up their market to non-EEC countries. The worries of the Germans, the other main objectors to the recommendations, were, however, removed. The Bundespost's ambitious videotext programme is being accompanied by a new law to extend its monopoly in new information systems and terminals such as modems, although this move will be examined by the European Commission's directorate on competition.

Jasper Becker

Chemical health risks

Not so great

Washington

After reviewing the scientific literature on the health risks associated with the use of nitrites and nitrates as food preservatives, a committee of the National Academy of Sciences National Research

Backing a winner

One of the few European research facilities which can claim a substantial lead over the United States in both size of budget and research quality, the high flux neutron beam laboratory at Grenoble (Institut Laue-Langevin, ILL), now has the money and agreements it needs to keep ahead of the opposition until the 1990s, its directors believe.

In the past two weeks two major decisions affecting the future of ILL, which provides services to 1,700 visiting scientists each year, have been taken. The treaty between France (the host country), Germany and the United Kingdom, which guarantees support for ILL, was extended a further 10 years to 1992; and the international steering committee, which decides the budget and a five-year forward look each December, has agreed to a substantial addition to the neutron source costing some £2 million, and an increase in the number of major instruments at Grenoble to make use of it.

The improvement — a new "cold source" which produces neutrons at long wavelengths for large-scale structural studies such as of polymers or of biological materials — was the principal part of a large programme approved in outline in 1979; but there were fears that cuts in German and British budgets might threaten it. These fears have now proved unjustified.

ILL directors see the new source, basically a tank of liquid deuterium which cools thermal reactor neutrons to low velocities and hence long de Broglie wavelengths, as a means of capitalizing on ILL's greatest successes (which have been in long-wavelength scattering) and of countering — or complementing — competition from accelerator-driven "spallation" neutron sources. These may provide improved flux and resolution at short wavelengths by making use of spallation reactions, where incoming protons (from an accelerator) collide with heavy neutron-rich nuclei (such as uranium) to produce a spray of neutrons ten to one hundred times the intensity of the incident proton beam. Spallation sources are planned in Japan, and at Argonne and Los Alamos in the United States; and one is under construction at the Rutherford Laboratory in the United Kingdom.

Robert Walgate

Council (NRC) has concluded that current exposure levels constitute an insufficient risk to require major reductions in their use.

However, because many nitrosamines and other nitroso compounds which can be formed from nitrates and nitrites have been found to cause cancer in laboratory animals, and can therefore be associated with cancer in humans, the committee has recommended some reductions.

The committee was set up by NRC at the request of the US Department of Agriculture and the Food and Drug Administration to study the health effects of nitrate, nitrite and *N*-nitroso compounds, following the publicity which occurred in August 1978 when workers at Massachusetts Institute of Technology found that sodium nitrite caused tumours in the lymph systems of rats.

At the time the federal government rejected calls for a temporary ban on the use of nitrites as a food preservative, but established an immediate study of the problem. In August 1980, the two agencies announced that they had found "insufficient evidence" to link the use of nitrites to cancer but said that they intended to study the issue further.

Last week's report is the first of two to be produced by NRC in response to the government's request by a panel chaired by Dr Maclyn McCarty of the Rockefeller University in New York. The panel's second report will consider current research and prospects for developing alternatives to nitrite as a food preservative.

In its first report, the committee says that the results of limited experiments suggest that nitrate is neither carcinogenic nor mutagenic, but that evidence from several epidemiological studies in human populations is consistent with the hypothesis that exposure to high levels of nitrate may be associated with an increase of cancer of the stomach and oesophagus, recommending further studies to confirm these preliminary findings.

The committee also says that scientific evidence does not indicate that nitrate acts directly as a carcinogen in animals. In contrast it says that most *N*-nitroso compounds are carcinogenic in laboratory animals, mutagenic in microbial and mammalian test systems, and that some are teratogenic in laboratory animals. However, it adds that such results are of limited value for predicting the quantitative risk to humans.

At the same time, it points out that nitrites found in cured meats account for only a small proportion of the total exposure to nitrosamines.

According to the committee, cigarettes represent the single largest source of nitrosamines, with a daily pack of American filter cigarettes producing an exposure of 17 microgrammes. In contrast, the level ingested from all dietary sources is about 1.1 microgrammes a day.

David Dickson

Satellite launcher market

Ariane set fair?

The European commercial satellite launcher business is itself well launched. With two successful test flights to its credit, Ariane, the European Space Agency's heavy satellite launcher, is already qualified for service. So whatever the outcome of the fourth and final test flight this week, the fifth launch next March will be the first of six promotional flights, after which production and marketing of subsequent launchers will be officially handed over to the French-based company Arianespace. But the space agency's role in rocket development continues. By 1983, Ariane 2 and 3, capable of placing 2,100 kg and 2,580 kg payloads into geostationary transfer orbit, will be in service.

Further ahead, in 1985 there will be a test launch of Ariane 4, which uses the basic design of Ariane 3 but is to be offered in six versions, with payloads of 2,300–4,300 kg, achieved by strapping onto Ariane 3 various combinations of liquid and solid-propellant boosters. This more ambitious version of Ariane 4 has been prompted by calculations of the mass of the telecommunications satellites likely to be built towards the end of the decade. The European Space Agency's swift approval of this version of Ariane 4 is evidence of its determination to compete internationally in the growing market for satellite launchers.

The only other present source of commercial launching facilities is the United States National Aeronautics and Space Administration. The Thor Delta and Atlas Centaur launchers can put 900 kg and 1,850 kg into geostationary transfer orbit respectively, compared with the current capacity of Ariane 1 of 1,700 kg. Although the shuttle will be capable of carrying much heavier payloads, Europeans are quick to say that it is technically unproven and that availability is still in doubt.

Meanwhile, there is little competition for Ariane from elsewhere. The Soviet Union has geostationary launch capabilities of 2,400 kg and 5,000 kg aboard Soyuz and Zonda, but these are not generally commercially available. Thus the most likely competition in the future will come from Japan, which already has a small rocket capable of launching 220 kg into a low circular orbit and is developing a heavy satellite launcher, which could rival Ariane, for a first launch in 1987.

The only other contenders so far are China, India and Brazil. China and India have already launched small scientific payloads into near-Earth orbits. The immediate goal of the Indian programme is to develop a Polar Satellite Launch Vehicle by the mid-1980s for putting meteorological and remote sensing satellites weighing up to 600 kg into near geopolar orbits which pass over all points on the Earth at the same time of day. Only then will attention be turned towards a launcher

for heavy communications satellites.

Brazilian plans are more tenuous. For a decade, the government has been talking of launchers to place remote sensing and meteorology satellites into orbit, though little seems to have been done.

Judy Redfearn

Polish science advice

Reforms in limbo

One of the casualties of the Polish government's sudden suspension of the Solidarity trade union at the weekend may well be the draft bill that would have made it obligatory for government agencies to consult "scientific experts" before making important decisions. News of the bill came in a communiqué issued last month after a meeting between the Prime Minister, General Wojciech Jaruzelski, the Primate of Poland, Archbishop Jozef Glemp and Lech Walesa, leader of Solidarity.

To be effective, the proposed bill would have had to ensure that consultations were more than an empty formality. In some cases it would seem, past governments acted without even an appearance of consulting scientists. A notorious case was the siting of vast industrial plants — the Skawina aluminium smelter and the Lenin steel mills — just outside Krakow. The decision to develop industry in the area in the 1940s had political overtones but meteorologically Krakow is a very poor site because of the limited circulation of the air. The toxic fumes from industry have undermined the health of the population and made the produce of farms unsafe within a radius of 50–60 km.

Until September 1980, any public discussion of such issues was impossible. Within a month of the signing of the Gdansk accords, however, a new Club of Polish Ecologists was established, based in Krakow, which early in January succeeded in having the obsolete production lines at Skawina (which had been emitting hydrogen fluoride) closed down. General Jaruzelski has been a strong supporter of moves to give scientists a louder voice, and he seems committed to continuing with consultations. But exactly who will be involved in any future talks is now unclear, as a new format is likely to emerge after a brief hiatus.

A second new bill now being prepared is aimed at reforms in the Academy of Sciences. The academic secretary of the academy is at present directly responsible to the prime minister and has himself quasi-ministerial rank. In recent months there has been a considerable movement within the academy to change this anomalous status by making the academic secretary responsible only to his fellow academicians. It seems likely that the prevailing "state of emergency" will mean a considerable weakening of provisions in the bill aimed at increasing the independence of the academy. **Vera Rich**

Creation on trial

Battle engaged

Washington

While a federal judge in Little Rock, Arkansas, listened to the closing arguments this week in a case claiming that a new state law which requires the teaching of "creation science" violates the separation of church and state, both sides in the dispute were already sharpening the arguments for the next round of what promises to remain an escalating battle of wits.

In Washington, the National Academy of Sciences held the first meeting on Monday of a committee made up of prominent scientists and legal advisers who will prepare a legal brief on the scientific status of the theory of evolution to be presented by the academy as an *amicus curiae* ("friend of the court") document, either to the Arkansas court if there is enough time, or in any future legal proceedings.

Meanwhile the creationists are working on a revised version of their "model bill" used as the basis of the Arkansas law passed in March, requiring equal efforts to be devoted to teaching the theory of evolution and creation science in school biology classes. The new bill is designed to meet some of the legal challenges thrown up in the Little Rock proceedings and elsewhere.

Whichever way the Arkansas verdict goes, there is a good chance that the fight will make its way up to the Supreme Court. And the supporters of creation science are unlikely to be put off by an adverse legal ruling, since they claim to be backed by a groundswell of popular support.

The academy panel is being chaired by Dr James Ebert, vice-president of the National Academy of Sciences and president of the Carnegie Institution of Washington. Others on the panel include Dr Steven Weinberg, professor of theoretical physics at Harvard; Dr Preston Cloud, professor of biogeology at the University of California, Santa Barbara; and Professor Norman Newell, curator emeritus in the department of invertebrates at the American Museum of Natural History in New York.

Several legal experts have been included on the panel to advise on the constitutional issues raised by the creationists. These include Mr Peter Barton Hutt, previously general counsel of the Food and Drug Administration, and Dr Richard Maserve, a staff member of the Office of Science and Technology Policy under the Carter Administration. Both are now members of the Washington law firm Covington and Burling.

The legal brief will concentrate on providing an academy-endorsed statement on the definition of science which, it is hoped, will help both courts and state legislatures distinguish the philosophical status of the theory of evolution from creation science.

The academy is also considering

producing a booklet to summarize current thinking on evolutionary theory.

The main claim being put forward by the state attorney general, Mr Steve Clark, in Little Rock is that, since holes can be picked both in the theory of evolution as a conventional science and in creation science as a conventional religion, the two are "just as scientific" and "just as non-religious" as each other.

The American Civil Liberties Union (ACLU), which has filed the case against the state of Arkansas on behalf of several local religious groups and school teachers, has so far had no difficulty in generating substantial support, from both the legal and the scientific professions, in preparing its case. A prominent New York law firm for example has been providing free legal support — including extensive research and the services of eight back-up attorneys in Little Rock — which would normally cost many hundred thousand dollars.



"Next witness"

ACLU has received help and advice from between 60 and 70 scientists in preparing a brief. Among those called to the witness box who gave a vigorous defence of evolutionary theory and challenged the claims of creation science were Professor Francisco Ayala of the department of genetics at the University of California in Davis, a member of the new National Academy of Sciences committee; Dr Gary Dalrymple, a geologist who is an associate director of the Western region of the US Geological Survey; and Harvard evolutionary biologist and historian Dr Stephen Jay Gould.

In contrast, although the state attorney general's office is presenting a number of scientists to put the creationist interpretation of human origins, few have significant standing in the scientific community. Attorney General Steve Clark complained last Thursday that several scientists had refused to testify in defence of the new law, suggesting that they had been subject to "peer group pressure".

Not all creationists have been happy with the way that their case is being handled. Two of the more prominent attorneys associated with the creationist movement, constitutional expert Wendell Bird and Virginia attorney John W. Whitehead, complained that they had been excluded

from playing a major role in preparing a defence of the law, and that the state had therefore prejudiced its own chances of defeating the law suit.

Both, however, are now preparing for the legal battles that lie ahead. And even if ACLU wins in Little Rock — as seems likely, given the liberal background of Judge William Overton and the way he has handled the case so far — next time round the arguments could be tougher to defeat.

One passage in the Arkansas bill, for example, speaks of creation science being based on the explanation that the world was created *ex nihilo*, a passage which theology professor Langdon Gilkey of the University of Chicago described as “the most religious” of various statements implying the involvement of a God, since “there are no other sources at work”. The new version of the model bill, however, which is being circulated by Mr Paul Ellwanger of Citizens for Fairness in Education of South Carolina, merely states that creation science must be based on “evidences that indicate creation of the Universe, matter and energy suddenly”.

Given the fact that challenges from creationists are unlikely to evaporate, even if they lose the Arkansas case, ACLU is asking the judge for a “finding of fact” that any science must be based on natural laws and must be explanatory, falsifiable and tentative, criteria which they hope will rule out creation science as a genuine science, however it is described.

David Dickson

UK cancer research

Unequal shares

If the Medical Research Council's (MRC) reshuffle in the allocation of funds for cancer research in Britain was intended as a bid to save money, it has been largely unsuccessful. The complete withdrawal of financial support from two cancer research institutes in Manchester and Glasgow has saved the council a paltry £0.5 million.

Instead, it seems, the changes — announced last month in the council's annual report — were aimed at a rationalization of administration in the Patterson and Beatson cancer research institutes in Manchester and Glasgow. Since 1970 the two independent laboratories have been financed by a joint committee of the Cancer Research Campaign (CRC) and the MRC, with the two bodies taking equal financial responsibility. “The MRC's decision to relinquish financial commitment to the CRC,” says Dr John Paul, director of the Beatson laboratories, “has made administration here much easier. Working for two paymasters can become complicated.”

The Beatson and Patterson laboratories may benefit from dependence on the publicly-supported CRC, but the Institute of Cancer Research in London (ICR) is left as pig-in-the-middle. It had been hoped

Painful adaptation at cancer institute

The problems of the British Institute of Cancer Research (ICR) stem largely from the events of four years ago, when its funds were substantially cut and put on a basis unusual for a British institute. The block grant to ICR has been tapering away for the past few years. From next April, research groups will have to compete exclusively for funds with other applicants to the Medical Research Council and the Cancer Research Campaign. To make matters more complicated, block funding of laboratory services has now also been withdrawn so that, for example, a research group which needs electron microscopy is now expected to obtain sufficient funds to pay for it as a properly costed service; consequently ICR will be able to maintain an electron microscopy facility only if there is sufficient demand and cash from the research units to support it continually.

Worse still, in the period between the sudden early retirement of the previous director of ICR, Dr Thomas Symington, in August 1977 and the appointment last year of Dr Robin Weiss, little had been done to adjust to the new circumstances. Indeed, ICR had gone on spending almost as though its total funds had not been cut by about 18 per cent, with the result that Dr Weiss inherited a deficit of about £1 million even though the Imperial Cancer Research Fund had donated about the same amount to help stave off the day of reckoning.

Since his arrival, Dr Weiss has had to clear the £1 million deficit and adjust to an annual budget that is now about £6.5 million — £4 million from the Medical Research Council and the Cancer Research Campaign, the rest from legacies, the National Health Service, endowments and granting bodies. About £400,000 a year has been saved by early retirements, the freezing of

vacancies and other means — for example, ICR no longer has laboratory cleaners. (Has anybody noticed?) About 45 members of staff have gone, a loss offset because Dr Weiss, with separate funds linked with his appointment, has brought in about 20 new people to establish a core of molecular and cellular geneticists.

But now, Dr Weiss and his newly appointed deputy director, Dr Tony Davies, have to find more savings. Unless some means can be found to spread the contraction over four years or so, by which time more natural or voluntary retirements will have done the trick, it looks as if there will have to be perhaps 50 compulsory redundancies.

One immediate threat is to the Division of Tumour Immunology under Professor Peter Alexander at the Sutton branch of ICR. As a result of recent site visits, the Medical Research Council and the Cancer Research Campaign have very substantially cut the funds of this division, leaving uncertain the future of all the tenured staff.

Dr Weiss is not convinced of the justification of this drastic cut when he sees little or no evidence that either the council or the campaign is cutting back on tumour immunology in their own units. By similar reasoning, he also fears for the future of radiobiological research at ICR. With the Medical Research Council about to continue its large Radiobiology Unit under a new director, and with the Cancer Research Campaign committed to its Gray Laboratory in Northwood, Dr Weiss wonders if they will see fit to continue support for radiobiology at ICR, in spite of its good record and close links with clinical radiotherapeutics. Dr Weiss holds that the institute is better placed to integrate radiobiology with other research activities than other establishments.

Peter Newmark

that the MRC could allocate further funds to the ICR because of its divorce from the other two laboratories. But in practice the council has stepped up its commitment to the ICR by just 10 per cent, leaving the funding shared 60:40 with the CRC. This year the council also gave the institute £0.5 million for equipment, but this cannot be guaranteed in the future, says the MRC.

Dr Robin Weiss, ICR director, maintains that the reshuffle has had no immediate effect on the institute. But he is concerned about the future: “With the present arrangement of joint funding, I sometimes worry that we become neither's ultimate responsibility.”

In this financial crisis, it would seem logical to predict that the CRC would be loyal to its wholly CRC-funded bodies and that the MRC's responsibility would rest preferentially with its own units. That

leaves the ICR in a rather vulnerable position. For there is no agreement, says Dr Weiss, that the financially healthier CRC should make good where the MRC falters.

Perhaps, though, the concern of the ICR for its future stems from its rough ride through the cuts of 1977. And the MRC is not last to admit that the financial status of the institute is far weaker than that of the Beatson or Patterson laboratories.

And while the CRC and MRC swear allegiance, one message emerges loud and clear, affirming Dr Weiss' concern. The CRC, dependent as it is on finance from the public, can in no way guarantee to act as back-up to the MRC with its government funding under continual threat. It is understandable that the ICR, funded by both bodies, should look nervously towards future government cutbacks.

Susan Douglas

CORRESPONDENCE

NIH guidelines

SIR — Comments are invited on two proposals for a major revision of the US National Institutes of Health (NIH) guidelines for research involving recombinant DNA molecules. The recombinant DNA Advisory Committee (RAC) has developed a proposed revision and recommended that it be published for comment. This proposal appears in the *Federal Register* dated 4 December 1981. The major features of this proposal are:

(1) The guidelines would cease to be mandatory and would become a voluntary code of standard practice. The following requirements would be eliminated: that institutions have an Institutional Biosafety Committee (IBC), that investigators obtain prior approval from the IBC before beginning certain experiments and that investigators obtain prior approval from NIH before beginning certain experiments. The section of the guidelines specifying that noncompliance could lead to loss of NIH funds would also be eliminated.

(2) Section III of the guidelines giving containment levels would be greatly simplified, and most experiments currently requiring P2 or P3 containment would be recommended at P1.

(3) The prohibitions section (I-D) of the guidelines would be eliminated, although two of the current prohibitions (I-D-2 and I-D-5) would be retained as admonishments. An alternative proposal appears as item 7 in the *Federal Register* notice of 7 December 1981. The major features of the proposal are:

(1) The guidelines would continue to be mandatory for institutions receiving NIH funding. Certain experiments would require prior review by NIH. Certain experiments would require prior review by IBC, and certain experiments would require notice to an IBC simultaneously with initiation of the experiment.

(2) Section III of the guidelines would be reorganized and simplified. All experiments would fall into one of four classes. Physical containment requirements for some classes of experiments would be lowered.

(3) Three of five current prohibitions (I-D-2, I-D-4, and I-D-5 in the current guidelines) would be listed in a new section that would continue to require RAC review and NIH approval before initiation. Experiments currently falling under prohibition I-D-1 and I-D-6 could proceed after IBC approval.

Copies of the above-mentioned *Federal Register* notices as well as a summary comparing the current guidelines with the two proposals are available from the NIH Office of Recombinant DNA Activities. Comments on these proposals should be directed to the attention of the Director, Office of Recombinant DNA Activities, National Institutes of Health, Building 31, Room 4A52, Bethesda, Maryland 20205, USA. These proposals and comments on them will be considered by the RAC at its next meeting on 8–9 February 1982.

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Laotian postscript

SIR — The clandestine US war against Laos during the Second Indochina War was for the United States substantially an air war — in terms of environmental impact almost entirely so. Its operational aspects have been well described¹. Laos received more than 2.2 million tonnes of air-delivered munitions, fully 31 per cent of the US air war against all Indochina². The massive wide-area saturation bombing from B-52 stratofortresses alone ultimately added up to 800,000 tonnes of bombs, accounting for 36 per cent of all such missions flown during the war throughout Indochina. The air war against Laos was carried out during the years 1965–73; the three peak years were 1969–71, which were almost equally intense and together accounted for 68 per cent of the entire US air assault upon Laos was in southern Laos (the panhandle), carried out primarily against the supply routes into South Viet Nam (the Ho Chi Minh trail). The remaining 30 per cent of the bombing was directed against northern Laos, to a major extent against the Plain of Jars in central Xieng Khouang province.

Herbicide chemical warfare attacks by the United States during the Second Indochina War were at one time or another directed against all four of the Indochinese nations of the time, overwhelmingly so against South Viet Nam and in only small but unknown amounts against the others. However, the United States has recently released some relevant information regarding Laos³. During

the years 1965–69, the United States flew some 100 anti-plant missions (over 400 sorties), with 1966, the peak year, accounting for 76 per cent of them. Altogether 1,600 cubic metres of herbicides were dispensed, mostly (83 per cent) Agent Orange. This Laotian spraying thus represented 2 per cent of the total Indochina effort. The area sprayed was some 67,000 hectares (not counting overlap), 0.2 per cent of the area of Laos. Most by far of this spraying occurred in southern Laos; and most of the spraying was for purposes of forest destruction to expose the Ho Chi Minh trail. A very small but unknown amount of the total spraying (perhaps 3 per cent, the proportion of Agent Blue dispensed) was devoted to crop destruction in the Plain of Jars and possibly elsewhere.

The Plain of Jars was especially hard hit during the war in terms of both social and environmental upheaval⁴ and is recovering extraordinarily slowly^{5–8}.

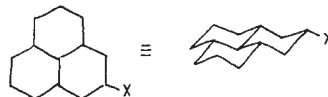
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To *trans*, *trans*, *trans*-tricyclo [7,3,1,0^{5,13}] tridecane

SIR — We are investigating the mechanisms of reactions of organic compounds in solution, in particular the relationship between a compound's reactivity and its molecular shape. We have prepared a number of new compounds, and the most recent of these has an especially pleasing shape and symmetry.



To celebrate this, we thought that a new form of reporting serious scientific results was appropriate, and the sonnet (Elizabethan rather than Petrarchan) seemed worth investigating. A fuller report, not in verse, is to be given today (17 December) at the Scottish meeting of the Perkin Division of the Royal Society of Chemistry at Strathclyde University.

Shall I compare thee to a single form
Of cyclohexane locked with bulky group?
Or should it be with that bicyclic norm
Whose ethane bridge 1,5 doth fix the hoop?
Thy undistorted, perfect, *trans*-fused rings
By force field calculation hold no strain;
No rapid rates which angle bending brings
As in bicyclo [3,2,1] octane.

Some help thy ground state conformation needs
To free an equatorial tosylate,
So ring-flip from an all-chair form precedes
Reaction from a boat transition state.

And then departs the leaving group when *trans*
Coplanar hydrogens the rate enhance.



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NEWS AND VIEWS

DNA supercoiling by DNA gyrase

from L. Mark Fisher

THE enzyme DNA gyrase is essential for bacterial viability and plays a critical role in DNA replication and repair, transcription and recombination. Since last reviewed in these pages¹, gyrase has been the subject of intense study and we are now beginning to understand how it carries out its function of catalysing the ATP-dependent introduction of negative supercoils — a coiling of the DNA helix axis itself — into circular duplex DNA.

Supercoiling can be understood in the following way. In linear duplex DNA, the two antiparallel DNA strands form a right-handed helix with about one helical turn for every ten base pairs — the B configuration. If the DNA is in the form of closed circles and has the same helix pitch as in the B configuration it is said to be relaxed. If, however, the two strands of the duplex circle are intertwined fewer times than in the relaxed circle, the DNA minimizes its departure from the energetically favoured B configuration by coiling about its helix axis (see Fig. 1). The degree of this supercoiling depends on the difference between the linking number (the number of times the two DNA strands are intertwined) of the DNA circle and the linking number of the same DNA in the relaxed state. By convention, underwound DNA forms 'negative' supercoils while overwound DNA forms 'positive' supercoils.

The mechanical strain energy stored in negatively supercoiled DNA exerts a persistent torsional stress tending to unwind local regions of the DNA double helix (Fig. 1). Thus, negative supercoiling catalysed by gyrase facilitates processes that entail unwinding of the helix, for example, at replication forks, during transcription and in the DNA strand transfer reactions involved in DNA recombination.

Gyrase belongs to a group of enzymes called DNA topoisomerases that change the linking number of circular DNA. They act by temporarily breaking and then reforming DNA phosphodiester backbone bonds. For all topoisomerases, transient DNA breakage is thought to proceed via the formation of a covalent enzyme-DNA intermediate that conserves the bond energy of the broken DNA phosphodiester bond for later rejoining. Topoisomerases

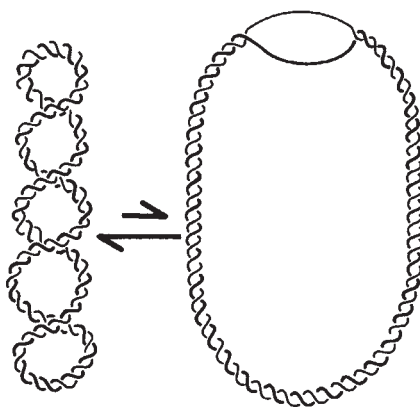


Fig. 1 Underwound circular duplex DNA (shown schematically on the right) forms negative supercoils (left).

are of two types, depending on whether they transiently break one DNA strand (type I) or both strands (type II)². The omega protein of *Escherichia coli* is an archetypal type I enzyme and the eukaryotic nicking-closing enzymes also belong to this class. In contrast, gyrase, phage T4 DNA topoisomerase and some recently discovered eukaryotic topoisomerases are type II enzymes². Gyrase is unique in catalysing DNA supercoiling: all other topoisomerases examined so far catalyse the removal of DNA supercoils, that is, they 'relax' DNA.

Two lines of evidence indicate that gyrase acts by making an enzyme-bridged transient double-strand break in DNA and passing another duplex segment through the break. First, gyrase can tie and untie knots in duplex DNA and can mediate the formation and resolution of catenated (interlocked) duplex DNA circles²⁻⁴. Second, both the supercoiling and ATP-independent relaxing activities of gyrase change the linking number of the substrate DNA in steps of two^{4,5}. This result is precisely that expected from passage of one duplex DNA segment through another (Fig. 2). Type I topoisomerases change the linking number of circular DNA in steps of one, consistent with mechanisms involving

single-strand breakage of DNA⁵.

How does gyrase catalyse DNA breakage? Information regarding this question has come from studies using the gyrase inhibitor, oxolinic acid. In the presence of the drug, *E. coli* gyrase forms a complex with DNA that on disruption with a detergent induces double-strand DNA cleavage at specific sites^{6,7}. Breakage of complementary DNA strands occurs with a 4 base pair (bp) stagger and results in covalent linkage of the two A subunits of the A₂B₂ gyrase complex, one to each 5' phosphate end of the break⁸⁻¹². The A protein-DNA linkage is via an O⁴-phosphotyrosine bond, the same linkage used by some type I topoisomerases in attaching to DNA^{11,13}. Thus, the gyrase A subunits are presumably engaged in breaking and resealing the DNA during catalysis, whereas the B subunits, known to bind ATP, participate somehow in energy transduction. Although the DNA sequences of many gyrase cleavage sites have now been determined, no readily apparent sequence rule governing cleavage has been uncovered⁸⁻¹⁰.

The work of Liu and Wang showed that when gyrase binds to DNA, there is a positive superhelical wrapping of DNA on the outside of the enzyme^{14,15}. Thus the gyrase-DNA complex resembles in part the nucleosome formed between histones and DNA, in which the DNA is known to be on the outside of the histones, although wrapped with a negative superhelical sense. Recently, detailed structural information about the gyrase-DNA complex has been obtained by several groups using nuclease protection methods^{9,10,16}. These studies made use of specific gyrase binding sites on DNA, which were identified using the oxolinic acid cleavage reaction, or in the case of *Micrococcus luteus* gyrase, by a DNA binding assay. Gyrase bound at these sites protects 100-140 bp of DNA against DNase I, contained within the 143 bp of DNA protected against staphylococcal nuclease^{15,17}. Protection is observed both in the presence and absence of oxolinic acid. A 40 bp region encompassing the oxolinic acid cleavage site is most strongly protected. Assuming that gyrase cleavage sites are sites of transient DNA breakage

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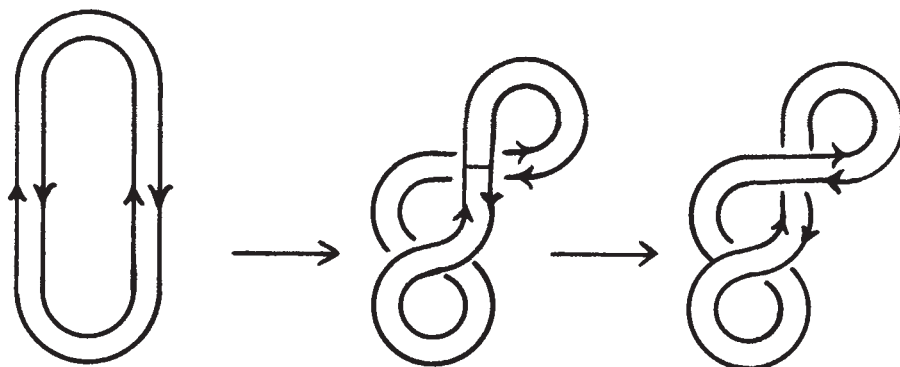


Fig. 2 Passage of one duplex DNA segment through another changes the linking number of circular duplex DNA in steps of two. A circular duplex DNA of unique linking number (left) is folded (centre) bringing together two different segments of DNA helix. Breakage of both strands of one helical segment, passage of the second helical segment through the break followed by resealing generates a DNA molecule (right) differing by two turns from the starting DNA. Thus, strand passage has changed the linking number of the DNA by two.

during catalysis, then this 40 bp segment presumably forms part of the gate through which the second DNA duplex is passed. Protected DNA flanking this region contains sites of enhanced DNase I sensitivity spaced on average 10 or 11 bp apart, in a pattern similar to that observed for DNase I digestion of nucleosomal DNA. This result is diagnostic of DNA adsorbed to a surface and indicates that DNA flanking the cleavage site is wrapped around the outside of gyrase.

Several related models incorporating double-strand DNA breakage have been advanced to explain how gyrase supercoils DNA. All of them embrace the following basic scheme. First, gyrase binds to DNA resulting in a positive wrapping of DNA around the enzyme and the formation of a compensating negative turn elsewhere in the DNA. ATP binding allows transient DNA breakage and passage of a second DNA segment through the break into the enzyme complex. The break is sealed and then ATP hydrolysis promotes release of the translocated DNA segment ready for another cycle. This sequence of

events has been deduced in part from the finding that the non-hydrolysable ATP analogue (β , γ -imido)ATP promotes only a single cycle of DNA translocation¹⁸. Wang *et al.* suggest that the positive superhelical wrapping of DNA on gyrase is retained during catalysis and that the translocated DNA segment can be anywhere in the nearby DNA¹⁹. Mizuuchi *et al.*⁴ and

Morrison and Cozzarelli¹⁶ propose that the translocated DNA segment is directly contiguous with the wrapped DNA. Strand passage then results in conversion of the wrapped positive DNA turn on gyrase directly into a negative turn, thereby accounting for the directionality of the supercoiling reaction. It is unknown how the DNA duplex passed into the gyrase-DNA complex during catalysis is subsequently released. The DNA could be shuttled along an interface between gyrase subunits ultimately emerging from the gyrase complex at a location different from its entry point^{4, 19}. Alternatively, a single entrance/exit port could be used, in which case, release of the passed DNA would require prior unwrapping of the resealed cleavage site¹⁶. These problems await clarification.

Type II enzymes have recently been isolated from eukaryotic cells^{20, 21}. Like T4 DNA topoisomerase, they do not supercoil DNA but act as ATP-requiring DNA relaxing enzymes. Intriguingly, they use ATP even though the removal of DNA supercoils is favoured thermodynamically. The biological functions of eukaryotic type II (and type I) topoisomerases remain to be elucidated. □

Site-specific recombination

from David Sherratt, Avril Arthur and Paul Dyson

GENETIC RECOMBINATION can conveniently be divided into two types distinguished by the extent of the DNA homology in the region of recombination. Homologous (or generalized) recombination occurs efficiently only between regions of DNA having at least several hundred homologous base pairs whereas non-homologous (or illegitimate) recombination occurs at regions of little or no homology. At least in *Escherichia coli* these recombination types are also distinguishable by the enzymes required: *in vivo* and *in vitro* experiments show that the RecA protein is directly involved in homologous recombination, whereas non-homologous recombination is RecA independent and is often promoted by proteins whose genes are adjacent to the recombination site. Non-homologous recombination can be accompanied by DNA replication, for example in genetic transposition¹, or can simply be a conservative reciprocal breakage and reunion of DNA strands as first proposed by Campbell for bacteriophage λ site-specific recombination in 1962^{2,3}.

Several recent publications show that site-specific recombination is utilized in prokaryotes for a variety of functions, and though different systems show some similarities, their underlying molecular mechanisms may differ to allow efficient

execution of their normal biological functions. The different possible outcomes of reciprocal recombination are schematically outlined in Fig.1, which also gives some indication of the activities and preferred directionality of the systems to be described here.

Bacteriophage λ integration and excision is the archetype of site-specific recombination and Campbell's model has been elaborated and substantiated by a wealth of elegant biochemical and genetic data⁴⁻⁶. Integration of λ *in vivo* requires λ int protein and normally occurs between *attP*, a region of about 240 base pairs (bp) on λ , and *attB*, a region of some 25 bp on the bacterial chromosome, generating two hybrid sites *attL* and *attR*. Though *attP* and *attB* are non-identical, they share a 15 bp homologous 'core' sequence within which recombination occurs. The reverse recombination reaction, between *attL* and *attR*, which leads to λ excision *in vivo*, requires both int protein and a second λ protein specified by the *xis* gene. *In vitro*, int promoted recombination occurs on negatively supercoiled DNA molecules containing both *attP* and *attB*, with little or no loss of supercoiling, no net DNA synthesis and no need for an exogenous energy source such as ATP. However,

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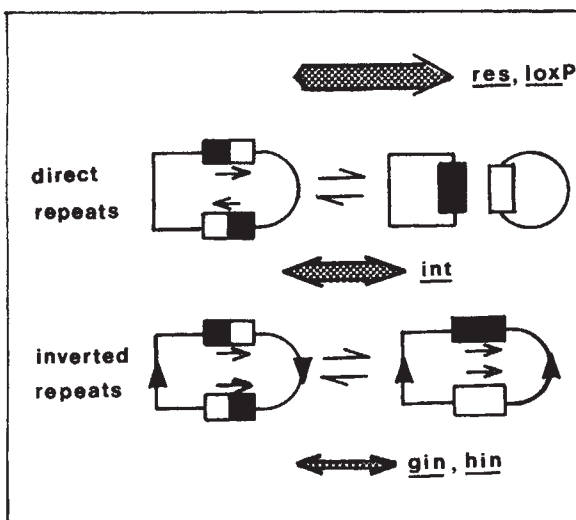


Fig.1 Consequences of intramolecular recombination between direct and inverted repeats. The hatched arrows give some indication of the activities and preferred directions of the different recombination systems. Whereas *int* protein acts on all combinations of substrates, *Tn3/γδ res* and *P1 lox* appear primarily to act intramolecularly on directly repeated substrates. The normal biological function of *hin* and *gin* is to act on inverted substrates. Note that if any recombination sites are symmetrical, that is, palindromic, then repeats of them would be in both direct and inverted orientation.

there is a requirement for a host-protein complex, though *int*-protein alone has topoisomerase activity, that is, it can mediate breakage-reunion reactions.

In contrast to λ -mediated site-specific recombination which can occur both intermolecularly (during normal integration) and intramolecularly (during excision), some other site-specific recombination systems, that have very different biological functions, usually operate intramolecularly at sites of fixed orientation. For example, in bacteriophage Mu, the expression of either of two different host ranges is specified by the orientation of an invertible segment of DNA bounded by a short inverted repeat. An adjacent gene, *gin*, specifies a recombination enzyme that acts at these repeats to mediate the inversion⁷. A closely related (and interchangeable) enzyme (*hin* protein) can similarly invert a 1 kilobase (kb) region of the *Salmonella typhimurium* chromosome by recombination across a 14 bp repeat to determine which of two flagellar types is synthesized⁷. In contrast to these two examples, where site-specific recombination acts to invert a sequence (exerting a 'flip-flop' phenotypic control), two other systems preferentially act on molecules containing directly repeated copies of the recombination site (Fig.1). Bacteriophage P1 exists as a plasmid in its lysogenic state and has a site-specific recombination enzyme which rapidly converts any dimeric plasmid molecules (generated during replication or homologous recombination) to monomers, by intramolecular recombination at a specific site, *loxP*⁸. It is postulated that such recombination may be important for stable maintenance of P1 as a plasmid, since its copy number control is so stringent that P1 replication is inhibited once there are two P1 replication origins in a cell. A single P1 dimer cannot be partitioned to both daughter cells.

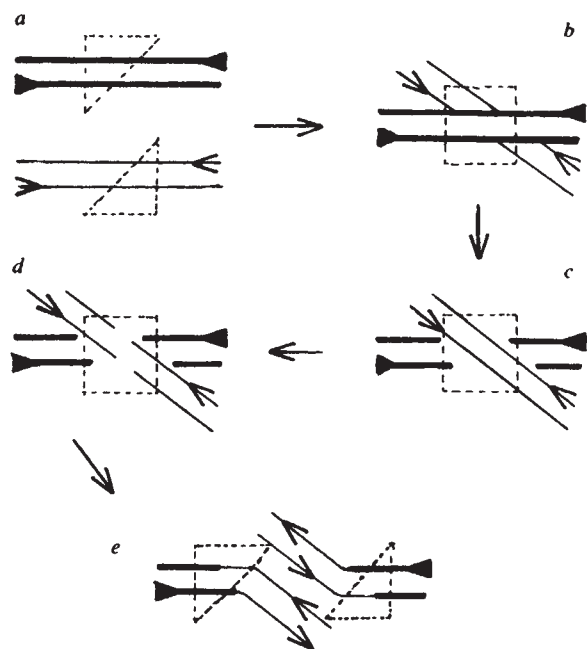
Transposons of the *Tn3* family also specify site-specific recombination systems that act most efficiently on directly repeated copies of the recombination site (*res*) within the same molecule⁹⁻¹¹. Such molecules are generated in the first step of

intermolecular transposition of these transposons and are rapidly resolved to the normal transposition end products by the site-specific recombination. Two recent papers by Reed and Grindley^{12,13} characterize the essentials of the reaction *in vitro* for one *Tn3* family member, $\gamma\delta$. The only requirements are the recombination protein (resolvase), Mg^{2+} and a negatively supercoiled circular DNA molecule containing two directly repeated copies of *res*. Although relatively large amounts of resolvase are required, the substrate is rapidly converted to the *in vitro* reaction products, two interlinked circles. In the absence of Mg^{2+} , a limited reaction occurs in which a resolvase-DNA complex accumulates. Removal of the protein from the complex with protease leads to the appearance of linear DNA fragments that are the result of double-strand breaks at each of the *res* sites. The breaks are staggered, having two nucleotide 3' extensions, and have remnants of the resolvase bound to the 5' ends. Formation of

such fragments depends on two *res* sites being present in the same DNA molecule, indicating that the breakage reaction can only be initiated on formation of a complex of resolvase bound to two *res* sites. If these resolvase-DNA complexes are subsequently incubated with Mg^{2+} , then some of them are converted to the normal *in vitro* products, supporting the idea that the staggered breaks are intermediates in the recombination. It therefore seems that $\gamma\delta$ resolvase, like known topoisomerases, can conserve polynucleotide chain phosphodiester bond energy generated during strand breakage and use it for subsequent strand rejoining. Although similar intermediates have not been reported in the λ *int*-promoted reaction, experiments have shown that five or seven nucleotide staggered breaks with 5' extensions occur during such recombination⁵.

Nash and co-workers have suggested that during λ recombination the two homologous 'cores' come together to form a four-stranded structure stabilized by intermolecular hydrogen bonds, a structure originally proposed by McGavin¹⁴. The *int* protein-promoted nicking of a single strand at the same position on each duplex could allow rotation around the uncut strands to bring broken ends adjacent to their recombinant partners, where ligation occurs. Subsequent nicking, rotation and ligation of the other two strands would produce recombinant DNA⁶. Though such a model is consistent with the type I topoisomerase properties of *int* protein, strand rotation appears not to be an inherent property of typical topoisomerases¹⁵. Reed's and Grindley's observations that $\gamma\delta$ resolvase can generate a double-strand cleavage could mean that this enzyme acts like a type II topoisomerase, the recombination occurring as one cleaved *res* site undergoes strand passage through the

Fig.2 A model for *res* recombination. (a) Two regions of DNA each containing at least a dimer of resolvase bound to *res* sites, (b) protein interactions bring the two *res* sites together and now (c) double-strand cleavage of one of the helices occurs and a double-strand passage event is initiated, as during type II topoisomerase action¹⁵. Strand separation and rotation are prevented by bound enzyme. During the strand passage, the second cleavage occurs (d) and is immediately followed by ligation to produce recombinant DNA. Though interlinking of helices to give catenanes will arise from accidental interwinding of DNA segments that will eventually reside in different recombinant products⁶, it could occur during strand exchange at the *res* site.



second (Fig. 2). Such a mechanism would implicate resolvase protein rather than four-stranded DNA both in initial synapsis and in the maintenance of superhelicity during recombination.

As more information becomes available it is becoming clear that despite similarities in these recombination systems, they have evolved diverse biological roles. For example, whereas the λ *int*, Tn3/ $\gamma\delta$ *res* and P1 *lox* systems may need to act very rapidly, inversion in the *gin* and *hin* systems occurs relatively rarely. This is to be expected, since there would be little sense in rapid switching from one host range or flagellar type to another. The relative efficiencies of these systems may reflect both the activities and amounts of the recombination enzymes and the nature and position of the normal recombination sites. Because the energy states of the reaction substrates and products ought to be very similar, the presence of a functional site-specific recombination system should generate an equilibrium mixture of similar quantities of reactants and products. Though this appears to be the case for the inversion in the 'flip-flop' recombination systems, the other systems have evolved ways of apparently shifting this equilibrium. If λ excision were simply a reversal of integration, then establishment of stable lysogeny would be difficult or impossible. The asymmetric λ recombination sites (*attP* and *attB*) generate different integration and excision substrates, which in turn allow the additional *xis* protein to be required for

excision. At first sight, the Tn3 and $\gamma\delta$ site-specific recombination reactions appear as if they should be readily reversible: in a molecule containing two directly repeated transposons there is at least 5 kb of homology surrounding the *res* sites, yet though such a molecule is an excellent recombination substrate, the reverse reaction to join two separate molecules through *res* recombination appears very infrequent. Perhaps even more surprising, *res* recombination *in vivo* or *in vitro* acts poorly on inverted copies of *res* in the same molecule. In contrast, λ *int* protein can act on *attP* and *attB* in all configurations (Fig. 1). We presume that topological considerations may determine the very different efficiencies with which *res* recombination takes place on the different substrates. DNA topology is likely to be important in control and determining specificity in many biological systems. □

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A plasma wake for Mars' satellite Deimos

from Alan Johnstone

SUCH diverse topics in space physics as the formation of the Earth's magnetosphere and the electrical charging of spacecraft can be gathered together under one heading — the interaction between a plasma and a moving body. The physical processes involved are quite varied and depend on the size of the body and its physical characteristics.

Although some experiments have been carried out in the laboratory, the conditions in space cannot be modelled accurately. Empirical knowledge of the interactions in space is therefore limited to observations of naturally occurring cases where the parameters cannot be varied in controlled conditions. Fortunately, the exploration of the Solar System in the last decade has revealed an unexpectedly rich variety of examples which cannot even be listed in a short article. Now, yet another one has been discovered. One of the martian satellites, Deimos, which is only 15

km across its largest dimension, is believed to create a disturbance in the solar wind which is 5,000 km across, 15,000 km downstream from the object (Bogdanov *J. geophys. Res.* 86, 6926; 1981).

The solar wind is an extremely tenuous gas of positively and negatively charged particles [10 per cm³] flowing outwards from the Sun at hypersonic speeds (400 km s⁻¹). The Russian spacecraft Mars 5, in orbit around Mars, found a region where the plasma density was lower than in the solar wind, the velocity was slower and the temperature higher at a time when the spacecraft was well upstream from any interaction with the planet itself. Another spacecraft, Mars 4, one million kilometres upstream from Mars at the same time, and which normally measured the same plasma parameters 20 minutes earlier than Mars 5, did not encounter a similarly disturbed region nor did it observe a variation in solar wind conditions which could have caused the martian bow shock to have formed in an unusual position upstream. Throughout its passage through the disturbed plasma

Mars 5 continued to measure the same strength and direction for the magnetic field as it had in the solar wind.

When the solar wind encounters a solid body which absorbs the particles that strike it, like the Moon, then it simply leaves a wake little larger than its diameter extending tens of diameters downstream. If Deimos created the effect observed by Mars 5 it must interact more strongly than this. If the body deflects the particles, and if there is a strong enough force between the particles to organize their motion in a collective way, then the solar wind will flow around the body with a fluid-like motion, thereby creating a much larger disturbance downstream. Since the average distance between collisions is greater than the distance from the Sun to the Earth, it cannot be a fluid of the type we know in everyday life. In fact, the magnetic field frozen in the plasma organizes the motion and is strong enough when the gyroradius of the ions is small compared with the scale length of variations in the flow. The solar wind can be deflected by a magnetic field but, since the gyroradius in the solar wind is about 100 km, Deimos would need a relatively large dipole moment to create an obstacle capable of deflecting the solar wind as a fluid. If Mars 5 had crossed a magnetic tail from Deimos then the magnetic field should have been aligned antiparallel to the solar direction. It was actually at an angle of 70° to this direction, so it is unlikely that the interaction is magnetic.

The flow can also be deflected by an atmosphere, as at Venus. There the upper atmosphere is ionized by sunlight and induced currents deflect the solar wind. The gravitational field of Deimos is far too weak to hold a permanent atmosphere but if there is a continuous outgassing from the surface then the density of the outward-flowing gas could perhaps be sufficient. The molecules do not interact with the solar wind until they have been ionized either by sunlight or by charge exchange. The interaction suggested by Bogdanov is one where the thermal pressure of the electrons created by photoionization is sufficient to balance the solar wind pressure. In order to obtain a fluid-like interaction the surface of pressure balance must be placed at least an ion gyroradius from the surface. The outgassing rate required to achieve this would result in the loss of one-third the mass of Deimos in a billion years. The outgassing could therefore be sustained for a period comparable with its lifetime.

Bogdanov compares this process with the interaction between a comet and the solar wind. Water, carbon dioxide and other volatile compounds sublime from the surface of the comet's nucleus as it is heated during its passage near the Sun. As this gas flows outwards from the nucleus it creates most of the familiar visible features associated with a comet. The outgassing rate from Deimos, required by Bogdanov's theory, is only one-millionth that of a large

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comet near perihelion so Deimos should not produce a large visible coma.

While Bogdanov's mechanism may be important near the body, the outgassing should be important over larger distances and near comets. When the neutral molecules are ionized by sunlight they are accelerated by the electric and magnetic fields up to the solar wind speed. This addition of mass to the solar wind flow requires a reduction in speed to conserve momentum. These implanted ions follow cycloidal paths around the magnetic field

direction and would not be detected by particle detectors aimed at the solar wind. The radius of the wake is roughly equal to the average distance travelled by a gas molecule before ionization. This mechanism should produce a larger wake at a lower outgassing rate.

Clearly the observations make Deimos a more interesting body but whether Deimos is the cause of the disturbance and, if so, which mechanism is responsible can only be determined by more measurements from spacecraft. □

atmosphere (so that effects of boundaries, for example, must be taken into account).

In contrast, it has been argued that for case B.1, instability is inevitable⁵, but that for case B.2 the stability of hydrostatic equilibrium solutions depends both on the ability of the loop base to adjust to coronal (or transition region) perturbations and on the response of the local heating process to imposed perturbations⁶.

Two very distinct approaches have been followed in trying to resolve this impasse. Recently, several groups have developed numerical time-dependent hydrodynamic codes which are capable of following the evolution of solar coronal structures; these studies follow the evolution of perturbed equilibrium models, and appear to conflict with much previous analytical work: imitating nature, the loop simulations show great robustness against perturbation¹³⁻¹⁵. The work of Craig and McClymont¹⁰ avoids the necessity for such 'brute force' calculations by asking a simpler (yet crucial) question: can the earlier analytical results predicting strong instability for fixed conductive flux conditions be maintained if one takes into account the rapid temperature variation along magnetic field lines at the photospheric base of the coronal loop structures; specifically, can a change in the (de)-stabilizing properties of the radiative energy losses at low temperatures significantly affect loop stability? This question's relevance is clear from the earlier results, which typically showed that the denser and cooler portions of the loop atmosphere tended to control the instability time scale⁵.

Starting with a realistic radiative energy loss function (in particular, at low temperatures), Craig and McClymont proceed to calculate the linear growth rate of the most unstable mode for a model with a fixed, vanishing base thermal conductive flux (case B.1 above), as a function of the amount of low-temperature material included at the base of the model atmosphere. The basic result is that, in typical solar conditions, the instability time

Thermal instability of solar loop structures

from Robert Rosner

THE past decade has seen a fundamental revision of our conception of the spatial structure of the multi-million degree plasma (corona) surrounding the Sun^{1,2}. The change involves recognition of the dominant role of the magnetic fields, which pass outwards from the solar interior through the visible layers of the solar surface, in structuring the overlying coronal atmosphere into regions topologically 'closed' and 'open' to the interplanetary medium.

The consequences for theoretical models of the solar corona have been quickly realized, primarily in calculations based on simple hydrostatic models^{3,4} involving magnetic plasma confinement. In the past few years, the existence and stability of these equilibrium solutions to the hydrostatic equations of motion have been extensively studied⁵⁻⁸. The extreme nonlinearities of the problem make the temporal behaviour of the confined solar atmosphere very difficult to predict theoretically. Especially troubling has been a discrepancy between theory and observation: most theoreticians believed that unless thermal conduction from the coronal loop down to the solar surface was 'large', instability was inevitable (so that the loop atmosphere was then unlikely to be in a state approximating hydrostatic conditions⁸), but X-ray observations of the Sun showed that the confined multi-million degree solar corona had little difficulty in maintaining plasma conditions often observationally indistinguishable from a quiescent, stationary state². The extent to which the predictions based on simple equilibrium loop models match the data is quite remarkable⁹. The disparity is of great general interest because related problems of thermal and magnetohydrodynamic stability have arisen in laboratory plasma studies and interstellar medium and extragalactic plasma astrophysics; the solar

corona thus provides a 'laboratory' in which plasma stability can be put to test. In a recent issue of *Nature* Craig and McClymont¹⁰ show how a large part of this difficulty can be resolved by more realistic modelling of the low-temperature boundary layer separating the coronal loops from the visible solar surface.

The early work on stability focused on two distinct issues: assuming isobaric perturbations, first, are the equilibrium solutions stable if the base temperature is held fixed; second, are the equilibrium solutions stable if the base conductive flux is held fixed. The first case (A) would seem to be appropriate if the base is located sufficiently deep in the atmosphere so that coronal (or transition region) perturbations do not disturb local energy balance. The second case (B) is more complex: the conductive flux may remain fixed either at a fixed spatial location (with varying base temperature; case B.1), or for a fixed temperature (with varying base location; case B.2); the latter case is appropriate if the base of the loop is chosen to lie in regions where radiative processes can control the temperature rise (see ref. 11), so that the base takes on the character of a free surface whose spatial location may vary. It is not obvious which of these three alternatives is in fact followed by the Sun's atmosphere.

Results for case A (which assumes unvarying conditions in the underlying chromospheric plasma) showed instability was inevitable if the base thermal conductive flux was very small ('thermally isolated' case), but with growth rates sensitively dependent on the ratio of base to peak temperature⁵⁻⁷. The basic physics of the instability involves the strong destabilizing influence of radiative energy losses (which decrease with increasing temperature for coronal plasma temperatures), and the stabilizing effect of thermal conduction, much as discussed in the classic paper by G. B. Field¹², but recast in the context of the strong inhomogeneities found in the solar

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scales for the unstable modes far exceed typical observed lifetimes (viz., days) of the loop structures in question; remarkably little low-temperature material is, in fact, required to quench the instability. Theory can thus now be fully reconciled with observation: if proper account is taken of the stabilizing influence of the boundary layer separating the solar corona from the underlying (visible) photosphere, analytical normal mode analysis does predict stability for time scales of the order of the observed coronal structure lifetime;

the error of previous calculations — and the source of the apparent discrepancy with observations — was simply the neglect of this stabilizing factor.

From both the observational and the theoretical perspectives, it thus now appears that the gross properties of the confined solar corona can be understood by considering simple hydrostatic equilibrium models, although the reasons for this are quite complex and, in some cases, are still not well understood⁹. However, when one looks at the corona in

some detail the simplicity disappears, and it is apparent that hydrostatic models are seriously inadequate. Observations of intensity fluctuations, of persistent up and down flows, of relatively cool matter residing at coronal heights, of apparent spatial mingling of hot plasmas at quite different temperatures, all call for more sophisticated modelling. Analytical methods seem inadequate for such modelling; the new plasma simulations have thus appeared at an opportune time. □

Hybridoma technology identifies protective malaria antigens

from F.E.G. Cox

VARIOUS partially successful attempts have been made to immunize experimental animals against malaria but preparations of the most easily obtained antigens, those derived from the forms of the parasite in the blood, have been relatively crude and poorly characterized. The use of monoclonal antibodies has now enabled scientists at the Wellcome Research laboratories to identify specific protective antigens and to use them to immunize mice against a virulent form of malaria¹.

A vaccine against malaria is one of the prime needs in tropical medicine and in a variety of models in laboratory animals vaccines have been prepared from sporozoites, the infective stages injected by the mosquito; schizonts, the dividing forms in the red blood cells; and merozoites, the products of this division that invade fresh cells. Monoclonal antibodies have been used to identify an antigen with a molecular weight of 44,000 that completely envelops the sporozoite and induces immunity to the rodent malaria parasite *Plasmodium berghei*². This antigen is specific to the sporozoite and seems to be a differentiation antigen that appears while the sporozoites are in the salivary glands of the mosquito and disappears soon after they have been injected by the mosquito and have entered the liver cells where they undergo their first phase of multiplication³.

Although a sporozoite vaccine is a logical and feasible possibility and would prevent the parasite from developing in the liver and thence the blood, a number of workers feel that should a single sporozoite escape the immune response, infection of the red blood cells would eventually ensue and therefore a vaccine should be based on the blood stages. The most promising candidate is the merozoite, and injections of merozoite antigens combined with adjuvants have been shown to protect monkeys against several species of malaria parasite⁴.

A monoclonal antibody against merozoite antigens of the rodent malaria parasite, *Plasmodium yoelii*, is known to confer protection on passive transfer⁵ and it has now been used to identify the antigen involved¹. The latter has a molecular weight of 235,000, is associated with an internal organelle of the merozoite and is specific to the merozoite. When mice were immunized with this antigen in Freund's adjuvant, they responded with high levels of merozoite-specific antibody and were protected from death following challenge, although they experienced relatively high parasitaemias lasting about 16 days. A second monoclonal antibody known to react with schizonts, but which was not protective on passive transfer⁵, was used to identify a second protective antigen. This is a surface antigen with a molecular weight of 230,000 which is present early in the maturation of the schizont and occurs on both schizonts and merozoites. Mice immunized with this antigen produced high titres of antibody against schizonts and were very resistant to challenge, experiencing only a mild infection lasting about 9 days. The significance of these results is that monoclonal antibodies can be used to identify protective antigens, but it is also interesting that the best protective immunity was produced in response to the antigen whose corresponding monoclonal antibody failed to provide passive protection.

What exactly is the nature of these antigens? Using similar virulent and avirulent forms of *P. yoelii*, Taylor *et al.*⁶ also have produced a range of monoclonal antibodies to sporozoites, schizonts and merozoites. Their antibody to merozoites was specific to merozoites but cross-reacted with those of several species, was associated with an internal organelle possibly involved with penetration into the red blood cell but was not protective. Epstein *et al.*⁷, using *Plasmodium knowlesi* in monkeys, have obtained three hybridoma lines that produce monoclonal antibodies against merozoites. All three agglutinate merozoites and two block red cell invasion. The antigen involved has a

molecular weight of 250,000 (or 240,000 if saponin lysis of infected cells is used) and cross-reacts with various strains of *P. knowlesi*. A fourth group of workers (Perrin *et al.*⁸) have also raised monoclonal antibodies to various stages including merozoites, this time to the human parasite, *Plasmodium falciparum*, in the mouse, a non-susceptible host. The merozoite antigens involved were surface polypeptides with low molecular weights, 36,000 and 96,000, and antibodies against these inhibited the development of *P. falciparum* *in vitro*. Antigens with low molecular weights like these must be viewed with caution, because Holder and Freeman also detected low molecular weight polypeptides associated with schizonts and concluded that they were proteolytic fragments of the 230,000 molecular weight antigen¹.

Thus a number of potentially protective antigens on the merozoite have already been identified using monoclonal antibodies. Some are surface antigens and some are internal, some cross-react with other stages or the merozoites of other species and there is also variation in molecular weight. It is likely, therefore, that a number of antigens are involved in the immune response to the merozoite alone and these may operate sequentially, concurrently or alternatively. Antigens associated with schizonts¹ and sporozoites^{2,3} have also been identified using monoclonal antibodies. It is going to be a long time before anyone identifies the most important protective antigens in a malaria parasite but in the meantime there will be a quantum jump from the use of crude homogenates as vaccines to cocktails of characterized polypeptides and these are much more likely to be acceptable in human medicine than anything in experimental use at the present time. □

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Which direction for ocean drilling?

from J.R. Cann

A recent conference* on ocean drilling (COSOD) enabled some one hundred and fifty geologists and geophysicists — including both those involved in the field as well as sceptics from other areas — to take a cool look at the long-term future of scientific drilling of the ocean floor. Of particular interest was the debate over whether the drillship *Glomar Challenger* should continue to be used or whether it should be replaced, at considerable expense, by conversion of the much larger *Glomar Explorer*.

Ocean drilling has been conducted since 1968 from the special dynamically positioned drillship *Glomar Challenger*, originally entirely USA funded, but expanded in the mid 1970s to include participation by the UK, France, Germany, Japan and the USSR. Currently the project costs \$21 million per year of which \$14 million is required to run the ship. The non-US subscription runs at \$2 million per year per partner, on top of which is the spending required to support the experiments connected with drilling.

Following a similar conference in 1976, plans were initiated to convert the six times larger *Glomar Explorer* from its original deep lifting role to a vessel for deep ocean margin drilling, carrying all the safety equipment necessary for drilling holes 6 km deep in water 3 km deep. Although at first it seemed that the *Challenger* and *Explorer* programmes might develop in parallel, progressively revised cost estimates showed that this was unlikely.

Scientific returns from *Challenger* increased in quality, especially with the development of the hydraulic piston corer that recovers almost undisturbed sediments from as deep as 200 m below the ocean floor, but financial pressures became greater. The planned *Explorer* drilling was too costly for the US Government to undertake alone, and under President Carter the joint government/oil company Ocean Margin Drilling Program was evolved. Eventually, after an initiative from the NSF to merge the two programmes, the oil companies withdrew from the *Explorer* program on 6 October this year. A number of detailed geophysical compilations for the areas selected for deep drilling (to be available next year) were carried out as well as various feasibility studies for the engineering involved. A Lockheed-Sedco venture is currently studying how best *Explorer* could be converted — without the expensive and complex safety systems necessary for really deep penetration in deep water.

COSOD, which was originally conceived as coordinating the *Challenger*- and *Explorer*-based drilling programmes, was left with two principal tasks, both of greatest importance. One was to examine the scientific justification for drilling beyond 1983, and the other to consider, if drilling was to continue, whether using *Explorer* or *Challenger* would be best.

A. Shinn, newly appointed director of the Office of Scientific Ocean Drilling at NSF, set the framework from the NSF point of view. He pointed out four options: (1) to terminate all scientific drilling at the end of 1983, when *Challenger* finishes her current phase of operations; (2) to extend the *Challenger* program for up to five more years; (3) to convert *Explorer* to a *Challenger*-like ship and use her instead of *Challenger*; and (4) to convert *Explorer* to a ship fully equipped for deep penetration in deep water as envisaged by the Ocean Margin Drilling Program.

Option (1) he presumed, correctly, would be rejected by the conference, and option (4) he regarded as too expensive to support, leaving options (2) and (3). He indicated that running costs for *Explorer* would be similar to or somewhat more than those of *Challenger*, but that conversion of

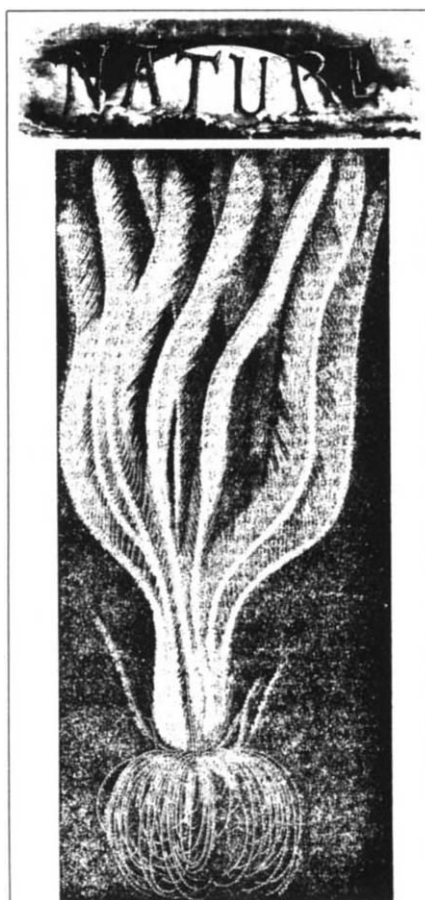
Explorer would put a halt to drilling (so that conversion could be funded) for about 18 months. He asked the conference to help decide between the two options, by assessing the ability of *Explorer* to handle larger amounts of drilling materials, to drill in high latitudes (especially in the Southern Ocean), to carry more scientists and to increase in drilling capability through a longer life, compared with the known and continuously available capability of *Challenger*.

A series of scientific reports and workshop discussions followed, including presentations from planning groups associated with both the present *Challenger* drilling and the Ocean Margin Drilling Program originally planned for *Explorer*.

On the final day the chairmen of the working groups presented their proposals to the reassembled conference, together with priorities for drilling. There were far too many proposals for excellent science than could be accommodated by one drilling ship. Among the highest priority targets presented, 'natural laboratories' was one watch word. These would consist of groups of holes in the ocean crust to study hydrothermal circulation, crustal construction and tectonics, which would act not just as sources of rocks for examination, but also as sites for *in situ* measurements and experiments, both in the short term and as long-term observatories. Work of this kind has already started, and shows great promise. Some holes would be sited on young, newly created crust where water at 350°C is seen to bring out sulphide ores from within the crust, and technical problems of drilling on bare, sediment-free rock, as well as at these very high temperatures, must be solved.

Testing the Vail Curve was another priority. This curve shows sea level in the geological past and was constructed by Peter Vail and his associates at EXON on the basis of seismic reflection profiles and drilling in shelf areas. Various aspects can best be tested outside traditional oil-producing areas, including the magnitude, suddenness and synchronism of the changes, and a number of such tests were proposed. If the Vail Curve can be made entirely reliable, then the consequences for understanding the geology of sediments would be great indeed.

Island arc regions, near the chains of volcanoes that border the Pacific and some other parts of the oceans, are places where plates are being subducted back into the Earth's mantle. They are still poorly understood, and are clearly of great importance in understanding global geology. Many kinds of targets were identified here, of which the most graphic is the drilling through the pile of sediments on the inner wall of ocean trenches into the descending plate of oceanic material,



Hairstar from the Taimur Coast.
From *Nature* 25, 22 December, 181 (1881).

*The 'Conference on scientific ocean drilling' (COSOD) was held in Austin, Texas on 16-18 November, 1981. The steering committee is preparing a report which will be available in the spring.

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where experiments could be performed. If much of the oceanic sediment is swept down into the mantle here, as seems increasingly likely, this could have profound effects on the chemical balance of the Earth's interior.

Palaeoclimates are inextricably linked with past oceanic circulation. In particular, the Antarctic regions carry the full record of glaciation and deglaciation, as well as an amplified record of pre-glacial climates. This is a prime target for any further drilling, but others too were identified in this area, such as the history of the vertical temperature and oxygenation structure of the deep oceans, and the nature of the great Pacific Ocean of 200 Myr ago. Only small fragments of this ocean crust remain, and these are buried deep beneath later sediments. However the Pacific then was even larger than today, and was essentially the only ocean, lying opposite the super-continent of Pangaea.

Finally there is the problem of the origin of split continental margins, such as those bordering the Atlantic. Since these areas are closely associated with petroleum generation and might contain deep-water petroleum deposits, they are clearly worth drilling. To understand them you need to drill where oil companies will not — on margins where sediments are thin and the history is close to the surface. During last summer great advances were made in our understanding of these areas by *Challenger* drilling around the Bay of Biscay and Rockall Bank, and this work is to be extended.

Which would be best for all this drilling, *Explorer* or *Challenger*? The tools and techniques people presented a report which essentially said that you get what you pay for. *Challenger* could be upgraded in many ways at a cost of \$7.5 million, although she could not apparently be fully strengthened for work in ice. *Explorer* could be converted for drilling at a greater cost, but would have greater capabilities, especially if extra money was available. Some of the scientific targets, such as the bare rock drilling, would require extensive engineering development, while others could only be reached using a fully converted *Explorer*. An informal vote was called for among the scientists present to see the extent of the support for *Explorer* or *Challenger*, and a substantial majority felt that the potential of *Explorer* outweighed the know abilities (and restrictions) of *Challenger*.

The general view of COSOD was that oceanic drilling should be regarded as integral a part of earth science as radiotelescopes are of astronomy. Instead of the present situation, where continuation of drilling has to be fought for every two years, there should be a general expectation, as with telescopes, that funding will continue and that the great contributions made to earth science by drilling will continue at the same or higher levels over at least the next decade.

Life seen from a medieval latrine

from Peter D. Moore

IF, as it is said, one can tell much about man's way of life from the contents of his garbage can, then one may indeed be able to tell even more from the contents of his latrine. It is perhaps surprising, then, that such depositories of environmental information have been rather neglected by archaeologists, despite their long-established affinity for middens. There are exceptions, of course, such as the delvings of Pike and Biddle (*Antiquity* 40, 293; 1966) into the sewerage system of medieval Winchester in search of human gut parasites and Dennel's (*Econ. Bot.* 24, 151; 1970) analysis of plant macroscopic remains from a sewer in medieval Plymouth. In the New World less is known but nevertheless, Schoewetter (*Archaeology of the Mammoth Cave Area*, Academic Press, 1974) has been able to analyse the contents of sub-fossil human faeces in Upper Salts Cave, Arizona.

A golden opportunity to develop an interdisciplinary approach to the study of long-abandoned lavatories arose in Worcester in 1975 when the clearance of an area of housing for the development of a new road revealed the existence of the lower part of a medieval barrel latrine filled with organic material. The find was excavated by Greig and its contents analysed for beetles (Osborne), plant macrofossils and pollen parasites (Greigh), bones (Jones) and cloth (Crowfoot and Raphael). It was radiocarbon-dated to the mid fifteenth century and the work has now been reported by Greig (*J. archaeol. Sci.* 8, 265; 1981).

The beetle fauna was dominated by *Tipnus unicolor* and *Mycetaea hirta*, which Osborne considers to be characteristic species of the cesspit habitat. He comments that the scarcity of these species today contrasts strongly with their abundance in medieval times, no doubt a consequence of such developments as the water closet. Of the other 36 beetle taxa recorded, an intriguing find is *Anobium punctatum*, the furniture beetle or woodworm. It may be that this hapless insect inhabited the (presumed) wooden seat above the latrine and that it died on emergence, but Osborne considers this taxon to be something of a coleopteran 'background noise' in a variety of deposits and therefore of little significance. Other beetles are characteristic of stored grain, or vegetables, which suggests an admixture of kitchen refuse in the barrel. It is possible

that such material could have been used for hygienic purposes together with the fragments of cloth and moss (tentatively identified as *Thuidium*) also found in the barrel.

Interpretation of the wealth of plant material found in the latrine is complicated by this input of extraneous vegetable matter. Undoubtedly some of the fruits and seeds found have passed through the human gut, such as *Robus fruticosus* (bramble — an unlikely candidate for hygienic uses), *Fragaria* (strawberry), *Vitis* (grape) and *Ficus* (fig). The figs must surely have been imported but it is possible that the grapes were locally grown. Many fruits and seeds were weeds of cultivation, or hedgerow species which could have found their way into collections of straw, indeed in the case of some of these, such as fennel (*Foeniculum vulgare*), coriander (*Coriandrum sativum*) and carrot (*Daucus carota*), it is difficult to be sure whether their origins are cultivated or casual. Certainly some of the seeds must be derived from the contaminant weeds of medieval crops, such as the corn cockle (*Agrostemma githago*), all species which have declined drastically in the present century with the advent of herbicides and whose demise is greatly mourned by the pestiphile lobby among conservationists.

Anyone who reads old herbals will be aware of the degree to which our forefathers were obsessed with the activities of their alimentary systems. It is natural, therefore, to look expectantly into the contents of the latrine in hope of finding direct evidence of the use of herbs. There are indeed some seeds of such plants as self heal (*Prunella vulgaris*) and black nightshade (*Solanum nigrum*), quite apart from the fig, of course. But some of the finds may give cause for concern, such as the deadly nightshade (*Atropa belladonna*) and water dropwort (*Oenanthe crocata*) and hepbane (*Hyoscyamus niger*). In view of the toxicity of these species, one cannot help wondering what eventually befell the user of the latrine.

A sad, though predictable, feature of the analysis is the high concentration of parasite eggs *Trichuris* (whip worm) and *Ascaris* (roundworm), with the former predominating in the ratio 3:1. This reflects the generally poor living conditions and lack of hygiene at that time.

From the contents of this barrel, Greig has managed to piece together an intriguing and complex picture of everyday medieval life in Worcester and may have purged some of the disinclination to exploit the archaeological potential of such residues.

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ARTICLES

Volcanic earthquakes at Pavlof Volcano correlated with the solid earth tide

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Volcanic earthquake swarms at Pavlof Volcano, Alaska, correlate significantly with solid earth tidal stress rate for periods just before and just after explosive eruptions. The correlation changes sign systematically during the course of the 1974 minor eruption sequence; pre-eruptive earthquake swarms occur during increasing tidal compression, while post-eruptive swarms occur during increasing tidal extension.

It has been proposed that the solid earth tide has a role in triggering both earthquakes and volcanic eruptions (see refs 1–5). Crustal stresses of the solid earth tide are of the order of 0.03 bar, with principal periods of ~ 12 h 25 min, 24 h 50 min, and 14.7 days, and constitute some of the largest pervasive short period oscillatory stresses in the Earth's crust⁶. We examine here the relationship between volcanic microearthquakes and the solid earth tide during the autumn 1974 minor eruption of Pavlof Volcano, Alaska. A correlation has been found between B-type⁷ volcanic earthquake swarms and solid earth tidal stress for periods of 3–4 days before and after eruptions. The correlation changes sign systematically for time periods before and after the eruptions, corresponding to periods of presumed inflation and deflation of the volcano.

Pavlof is a 2,715-m high stratovolcano located near the end of the Alaska Peninsula at 55°25' N, 161°54' W. It is one of the most active volcanoes in North America with at least 27 documented eruptions since 1700 (S. Hickman, personal communication). Strombolian eruptive activity typically includes lava fountaining and flows, emission of steam and ash, and minor explosions. Analysed lavas are basalt, with small phenocrysts of plagioclase, olivine, hypersthene and augite⁸. The upper portion of the mountain is covered by glaciers.

Twelve seismometers are in operation near Pavlof (Fig. 1). BLH and PVV are stations of the Shumagin Islands regional network, operated since 1973 by Lamont-Doherty in cooperation with the University of Alaska. The remaining local network stations were installed by Lamont-Doherty in 1976, that is, after the sequence of eruptions discussed here. Most of the data presented here are from station PVV, a short period (1 Hz) vertical seismometer located adjacent to an old lava flow 7.5 km south-east of the volcano's summit. The data are recorded on paper heliorder records.

The autumn 1974 eruption sequence began with minor explosive activity from 2 to 21 September, followed by a period of repose. Explosive activity occurred again from 29 October to 19 November, 25 November to 16 December and 25 December to 6 January. Each of these three periods of explosive volcanism began 3–4 days after fortnightly neap tides, in close agreement with Johnston and Mauk's previous results from Mt Stromboli, Italy⁹.

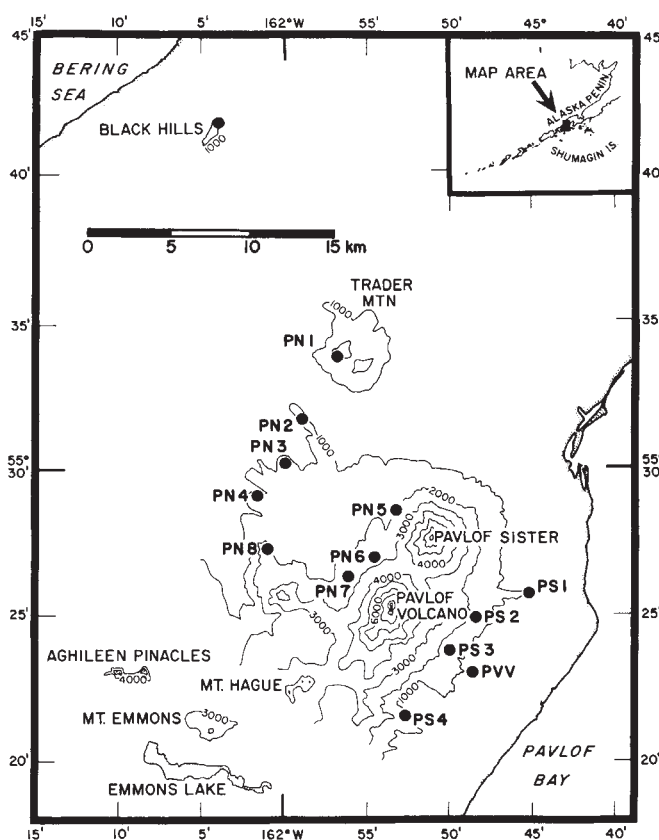


Fig. 1 Map showing stations of the Pavlof seismic array. Data are from station PVV, 7.5 km to the south-east of the volcano's summit.

Table 1 Pavlof volcano seismicity

Period	Mean no. of events per 2 h	s.d.	No. of data points
Non-eruptive	10.05	5.65	96
Pre-eruptive	26.74	18.81	114
Eruptive	20.24	13.58	178
Post-eruptive	14.19	13.53	161

Seismicity

Data reduction consisted of detailed counts of each of four different types of volcanic earthquakes: high frequency tremors, harmonic tremor, explosion earthquakes and B-type earthquakes ranging in magnitude from -0.2 to 1.0 (Fig. 2) (S. McNutt in preparation). The numbers of events of each type per 2-h interval were plotted on histograms (see Fig. 3) for the entire time period from 1 September 1974 to 30 April 1975. Table 1 gives statistics for the general seismicity, broken down into four periods: non-eruptive (background), pre-eruptive, eruptive and post-eruptive. Seismicity is highest during pre-eruptive periods, followed by eruptive, post-eruptive, and background periods.

Table 2 Number of B-type events versus tidal stress rates in certain azimuths

Azimuth		0°	45°	90°	135°	Volumetric	N
Non-eruptive	14–16 January	0.17	0.19	0.19	0.14	0.20	36
		–0.21	+0.04	–0.46	–0.22	–0.26	
Eruptive	4–8 November	0.02	0.11	0.09	0.00	0.05	42
		–0.04	+0.01	–0.26	0.00	–0.11	
Pre-eruptive	29 October–1 November	0.45	0.49	0.49	0.41	0.51*	37
		–1.24	–1.67	–2.48	–1.45	–1.50	
Post-eruptive	18–21 November	99%	99%	99%	99%	99.9%	46
		0.22	0.22	0.33	0.31	0.29	
Pre-eruptive	22–26 November	+0.50	+0.56	+0.86	+0.72	+0.56	43
		—	—	98%	95%	95%	
Post-eruptive	7–12 January	0.25	0.32	0.38	0.26	0.33	60
		–0.82	–1.25	–2.16	–1.05	–1.10	
		—	95%	98%	—	95%	
		0.35	0.36	0.34	0.32	0.37*	
		+0.93	+1.09	+1.26	+0.98	+0.93	
		99%	99%	99%	98%	99%	

The upper value of each entry is the correlation coefficient, the second is the normalized slope and the third is the confidence level estimate for those cases where it is greater than 90%. *N* is the number of data pairs used.

* The data plotted in Fig. 4.

Eruptive periods are so designated on the basis of the presence of explosive activity.

B-type earthquakes (Fig. 2) at Pavlof are nearly identical to those observed elsewhere^{7,10}. They have emergent onsets, no clear S-phases, dominant frequencies of ~ 1.4 Hz, and coda durations of ~ 20 s. Other studies in Japan⁷ have located B-type earthquakes at extremely shallow depths (1 km) immediately adjacent to active vents. The Gutenberg–Richter magnitude frequency relation for 1,316 B-type earthquakes which took place from 1 to 8 December has a negative slope or *b*-value of 2.6 ± 0.2 , in close accord with values calculated for other volcanoes^{7,10,11}. Explosion earthquakes (Fig. 2) closely resemble B-type earthquakes, except for an impulsive spike ~ 21 s after the signal onset. This spike is the air shock phase.

Tidal computations

The earthquake histograms were compared with a synthetic solid earth horizontal stress tide in azimuths 0° – 180° and with volumetric stress. The components of the tide as a function of time and position were calculated by Longman's method¹², using a program which agrees closely with independent computations. Longman's method calculates tidal potential, from which stress is easily derived by differentiating and using the stress–strain relations with standard values of Love numbers and Lamé parameters. Visual inspection showed that earthquakes tended to occur at times of increasing or decreasing stress in certain azimuths. We therefore differentiated the synthetic stress tides with respect to time to generate stress rate tides (Fig. 3).

The ocean loading tide must be accounted for as well as the solid earth tide; for the particular case of Pavlof, the ocean load stress tide is approximately in phase with the solid earth tide. A rough calculation using Farrell's¹³ Green's functions and Schwiderski's¹⁴ ocean tide charts shows that for volumetric stress and for stress in azimuth N90° E the load tide maxima are within 1 h of the solid earth tide maxima. The load tide amplitude is $\sim 30\%$ that of the solid earth tide; hence the inclusion of the load tide changes the phase by <1 h. We do not consider here the detailed effect of topography on the stress tide, but note that topography normally has a small effect on the phase¹⁵.

Correlation data

Linear correlation coefficients between number of earthquakes per 2 h period and tidal stress rate for 15° increments in azimuth were calculated for portions of the eruption sequence. Figure 3 shows the number of earthquakes and two components of stress rate plotted against time for two pre-eruptive and two post-eruptive portions of the data. The third post and pre-eruptive

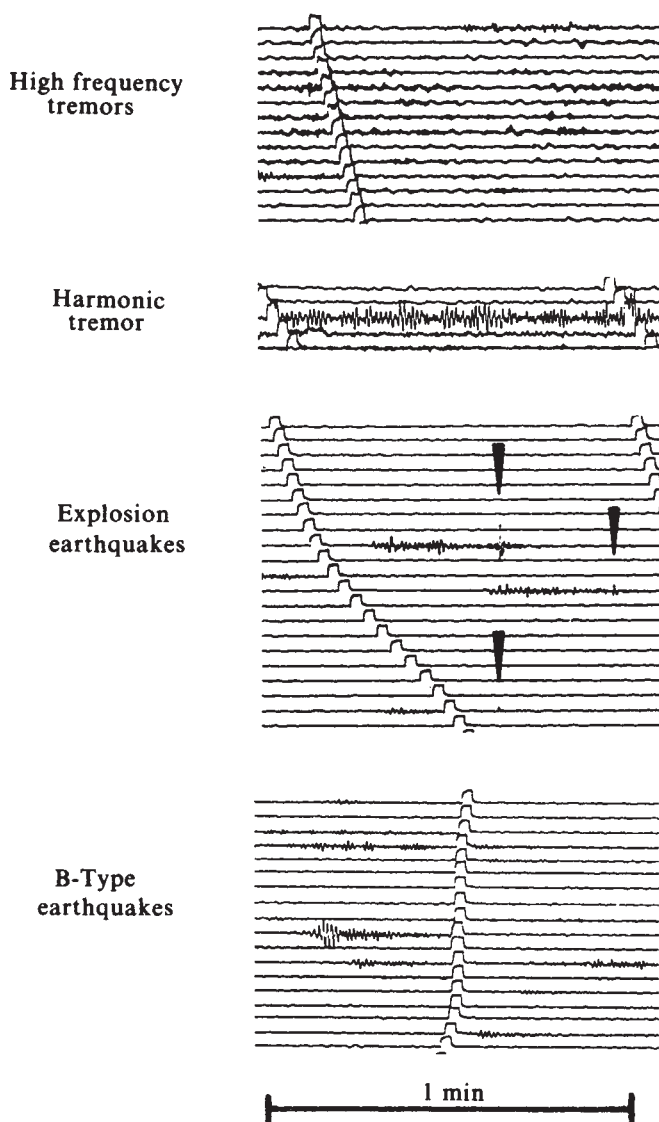


Fig. 2 Types of seismic signals recorded on helicorder from station PVV. Arrows point to air shock phase of explosion earthquakes. Note the similar frequency content (≈ 1.4 Hz) of B-type earthquakes, harmonic tremor, and ground waves from the explosion earthquakes.

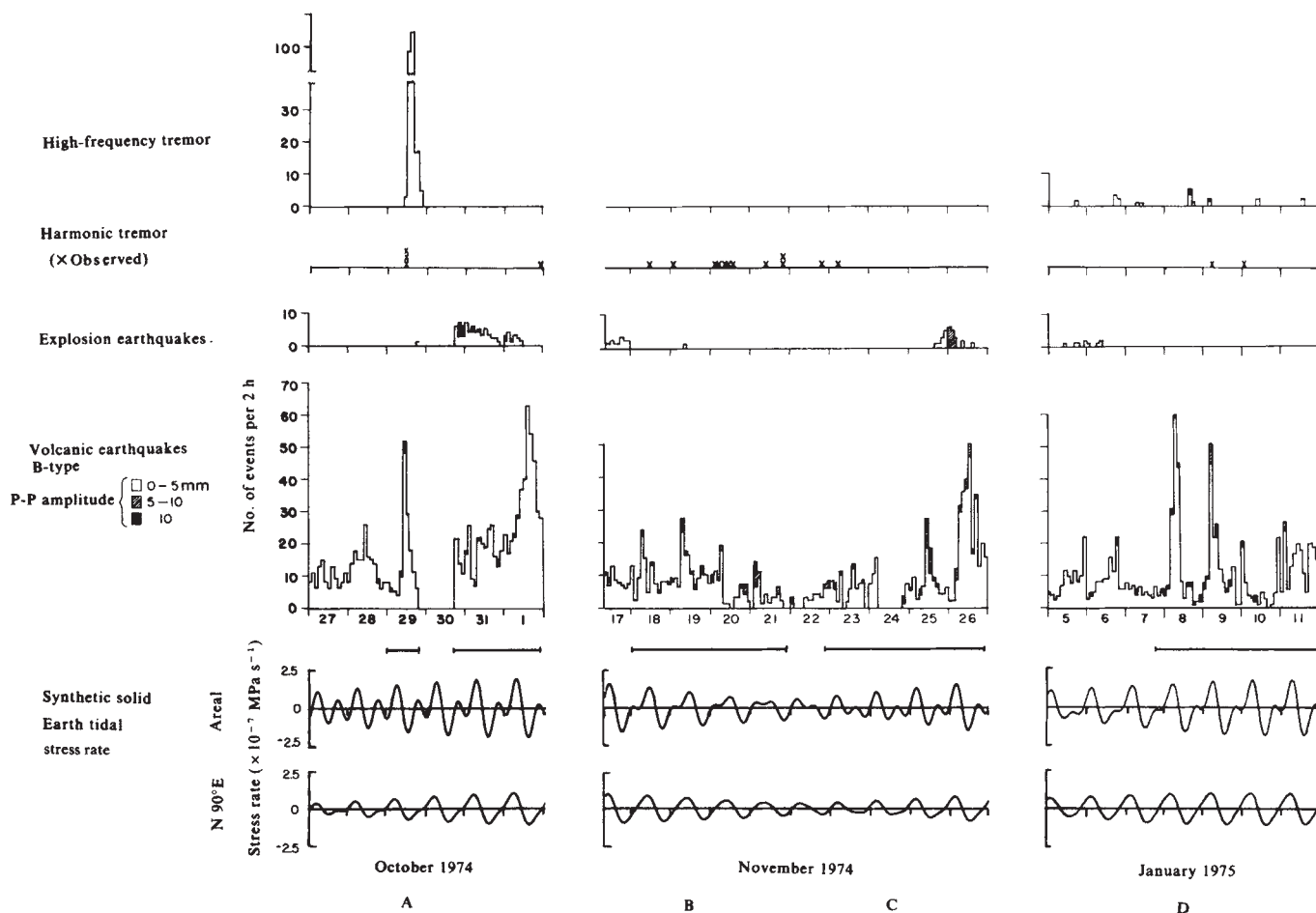


Fig. 3 Portions of histograms showing numbers of volcanic earthquakes of each type compared with synthetic solid earth tidal stress rate. A positive stress rate indicates increasing extensional stress. Note in particular the relation between the number of B-type earthquakes and tidal stress rate. A and C label pre-eruptive periods, while B and D label post-eruptive periods. During periods A and C peaks in the number of B-type earthquakes tend to occur at negative peaks in stress rate, while during periods B and D earthquakes occur at positive peaks in stress rate. See text for fuller explanation. The horizontal bars between the histograms and the tidal data show the portions of data actually used in the correlation calculations.

sequence is not shown; seismicity during this time period was the highest for the whole 7-month study period, and for reasons given below the results are less reliable. The results given in Table 2 show no statistically significant correlation with tidal stress rate for either non-eruptive or eruptive seismicity. However, pre-eruptive and post-eruptive seismicity do show statistically significant correlations between the number of B-type volcanic earthquakes and the solid earth tidal stress rate for certain azimuths (Fig. 4). During pre-eruptive and post-eruptive periods earthquakes tend to occur at the times of tidal zero-crossings, that is when the rate of change of the tidal stress is most rapid. During pre-eruptive periods the correlation is negative: B-type earthquakes tend to occur at the time of most rapid increase in compressional tidal stress. For post-eruptive periods the correlation is positive: earthquakes tend to occur at the time of most rapid increase in extensional tidal stress. Table 2 shows a small but representative sample of the computed correlation data: estimates of correlation coefficients of regression, normalized slopes (that is, numbers of earthquakes per unit stress rate) and the confidence level of the correlation (the probability that the true correlation is greater than zero) are listed. We find that correlations with horizontal stress rate are better for azimuths from 75° to 105° than for other azimuths; correlations with volumetric (or areal) stress are similar to the best horizontal stress correlations.

Discussion

Ryall *et al.*¹⁶ stated three criteria that should be met to evaluate optimally a tidal correlation: a statistically significant number of

events, a limited focal region, and a relatively constant character of accumulated strain in the region, as evidenced by focal mechanism study of the earthquakes. The first two conditions are met at Pavlof Volcano. Focal mechanisms could not be determined for the volcanic earthquakes because (1) it is nearly impossible for volcanic earthquakes to determine polarity of the emergent first arrivals, and (2) only one station was in existence at the time of this sequence of events; however, the signal characteristics are remarkably similar and are consistent with a relatively constant mechanism. Several other factors are important: the eruption was small, hence processes were smooth and slow rather than catastrophic; except for one time period (16–25 December) the seismicity was low enough that there was virtually no overlap problem in reading records; and the earthquakes themselves were small, thus the tidal stresses may have attained a larger fraction of the presumed stress drops ($\approx$ a few bars) of the earthquakes than is the case for catastrophic events.

Our data are insufficient to determine unambiguously the focal process responsible for causing the earthquakes. Two processes are most likely; mode I (tensional) cracks and mode II (shear) cracks, which are both thought to occur as a result of magma injection into the volcanic pile. For the same crack geometry, however, the two processes would require different orientations of the principal stress axes to enhance most crack growth, or, vice versa, for a given stress system the two types of cracks most likely to be activated would be oriented at different azimuths. Our correlation data suggest a preferred orientation of cracks, but we cannot determine this orientation uniquely. Determinations of maximum horizontal compressive tectonic

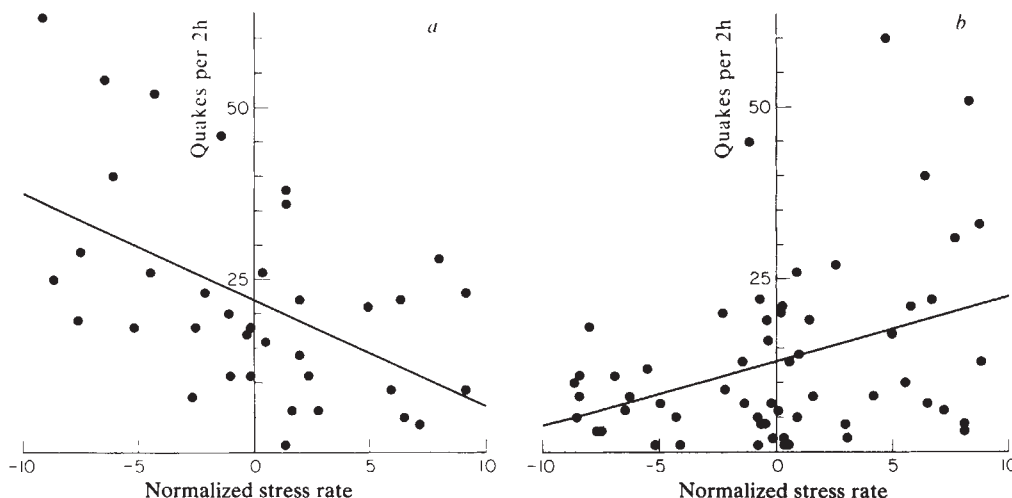


Fig. 4 Linear regression fits between earthquake frequency and stress rate. *a*, The data for a pre-eruptive period where the correlation is negative. The correlation coefficient is 0.51, and is >0 at the 99.9% confidence level. *b*, A post-eruptive period where the correlation is positive. The correlation coefficient is 0.37, and is >0 at the 99% confidence level.

stress^{17,18} and the azimuth of relative plate convergence of the North American and Pacific plates¹⁹ suggest an azimuth for the P-axis of maximum compression of approximately 150° which may control preferred crack growth in this direction if tensile cracks dominate, or at some angles $\pm 45^\circ$ from this azimuth if shear cracks dominate. This discussion does not consider pore pressure variations, which could change the effective stress significantly.

We tentatively interpret the change in sign of the correlation in terms of inflation and deflation of the volcano before and after eruptions, respectively. Some volcanoes tend to inflate before and deflate after eruptions^{7,20}, presumably due to buoyant forces exerted by magma on the volcanic edifice as the magma migrates through dykes, but no geodetic data are available for Pavlof to confirm this hypothesis.

The tidal correlation applies only for a period of 3–4 days before and after explosive eruptions. As in previous studies²¹ this implies that the volcano is sensitive to changes in ambient stress rate of $\sim 2.5 \times 10^{-7} \text{ MPa s}^{-1}$ during the times when magma

is near the Earth's surface. Other volcanoes have quite slow rates of tectonic stress accumulation before eruptions²⁰. The time period of 3–4 days may represent the time of magma ascent from or descent to a shallow magma chamber.

Our results indicate that the earth tide may have an effect on certain aspects of volcanic activity at Pavlof. This paper is believed to be the first to show a tidal correlation whose polarity with respect to tidal phase varies systematically during a volcanic eruption.

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Lithospheric studies based on holographic principles

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Earth holography was applied to Norwegian Seismic Array (NORSAR) earthquake P-wave recordings. Simulation of the NORSAR sensor configuration indicates that a satisfactory reconstruction of the essential features of an embedded body is feasible with a depth resolution which seems to be better than that attained using more traditional time and amplitude inversion schemes. The earth holography concept was tested using NORSAR recordings of three deep earthquakes at teleseismic distances. The results indicate lithospheric heterogeneous bodies with a significant vertical extent. The most pronounced scattering features of these inhomogeneities are located at depths around 100, 148 and 212 km.

DETAILED mapping of subsurface geological structures is important both for exploration for natural resources and for a better understanding of tectonic processes. The best tools are those tied to seismic wave analysis in view of the very short

wavelengths involved compared with other geophysical investigations. During the 1970s two novel approaches were introduced: (1) the scattering approach using the concept of an isotropic random medium with a prescribed statistical velocity

perturbation, correlation distance and turbidity factor¹⁻³; and (2) the ACH method⁴⁻⁸ which inverts travel time observations of a deterministic model into velocity perturbations. These approaches proved successful both in terms of three-dimensional seismic images of subsurface structures and in explaining precursor wavetrains⁹ in the seismogram. A viable alternative might be to introduce a deterministic scattering model, assuming the presence of discrete inhomogeneities.

The surface wavefield manifestations of such inhomogeneities are in terms of diffraction effects which leads to an inverse diffraction problem for their mapping. This is analogous to the image reconstruction problems of holography^{10,11}.

In applying holographic principles to analysis of seismological observations (earth holography), direct travelling seismic waves should be used because inhomogeneities often cause strong forward scattering. For example, Fig. 1 shows what the image of different structures should be when 'seen' by passing seismic waves. Compared with optic and acoustic holography, earth holography poses practical problems: complicated structure, the scale of the heterogeneities relative to that of useful seismic wavelengths is small, aperture of the sensor array is only of the order of 10–20 times the seismic wavelength as compared with a factor of 10^7 in optics, relatively poor wavefield sampling and background noise.

We now describe an attempt towards applying the principles of holography on a seismological scale. To test thoroughly the impact of the limiting factors an ultrasonic modelling experiment was undertaken to simulate the sampling of the NORSAR array in Norway¹² comprising 132 short period vertical seismometers (up to 1976).

Theoretical background

Despite extensive applications of the principle of wavefield reconstruction, its mathematical foundation has been formulated only recently^{11,13-15} and the wavefield reconstruction has been reduced to a boundary value problem associated with the scalar wave equation.

The solutions of the forward and inverse diffraction may be written in similar forms using appropriate Green's functions:

$$U(x_2, y_2, z_2) = \frac{1}{2\pi} \iint_{-\infty}^{\infty} U(x_1, y_1, z_1) \frac{\partial}{\partial z_1} \times \left(\frac{\exp[-jkr]}{r} \right) dx_1 dy_1 \quad (1)$$

$$U(x_1, y_1, z_1) = -\frac{1}{2\pi} \iint_{-\infty}^{\infty} U(x_2, y_2, z_2) \frac{\partial}{\partial z_2} \times \left(\frac{\exp[jkr]}{r} \right) dx_2 dy_2 \quad (2)$$

where for the forward problem the distribution of U (pressure or velocity fields) at plane z_2 results from the knowledge of the distribution in the z_1 plane $U = U_1(x_1, y_1, z_1)$, with $z_2 > z_1 > 0$. In the inverse problem the distribution of the wavefield in plane z_1 is determined from knowledge of the field distribution on plane z_2 (equation (2)). k is the wavenumber of the U -field and r is the distance between points in planes z_1 and z_2 .

A possible computational procedure for solving equation (2) follows from the convolutional nature of this equation, and is based on the FFT algorithm. Another technique which is adopted here is to take the derivative under the integral of equation (2) and assuming $k \gg 1/r$ we then get:

$$U(x_1, y_1, z_1) = -\frac{j}{\lambda} \iint_{-\infty}^{\infty} U(x_2, y_2, z_2) \frac{\exp[jkr]}{r} \times \cos \theta dx_2 dy_2 \quad (3)$$

where θ is the angle of incidence. The approximation of equation (3) in combination with equations (1) and (2) is reasonable for the present models.

Further simplifications are feasible by a series expansion of r in the exponent of equation (3) and by retaining only the first two terms. This would be analogous to the Fresnel approximation used for solving forward diffraction problems.

Ultrasonic modelling experiment

The set-up of the ultrasonic modelling experiment is outlined in Fig. 2a, where the 'transparent' or homogeneous body is a parallelepiped made from epoxy with an embedded aluminium cross serving as the inhomogeneity whose image is sought reconstructed by holography. A monochromatic wave frequency of 880 kHz was used which is equivalent to ~ 1.8 Hz in the seismological case. The choice of the frequency 1.8 Hz was motivated from the modelling experiment itself, implying an upper limit of 1.3λ for grid spacing. This is equivalent to ~ 4 km in the seismological case (using a mantle velocity of 8.2 km s^{-1}) which in turn reflects the NORSAR subarray sensor inter-spacing of 2–4 km. The major modelling parameters are also

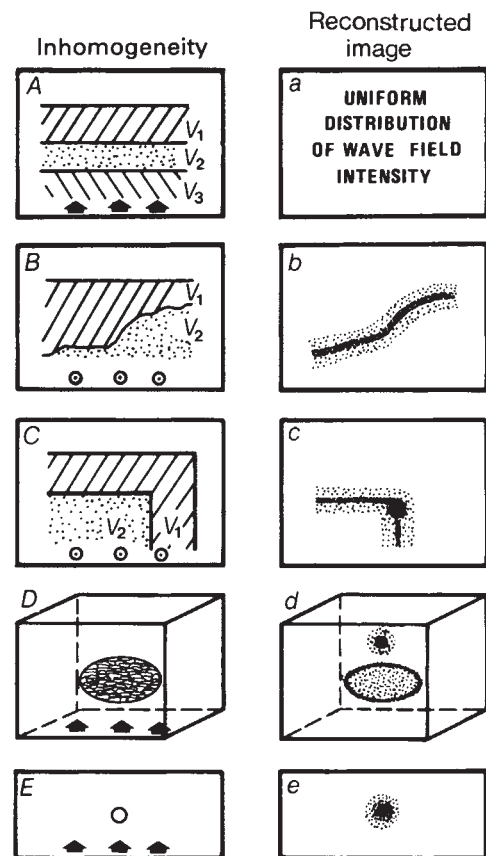


Fig. 1 Realistic earth inhomogeneities and their anticipated reconstructed holographic images. For A, D, E the illuminating plane-wave source is beneath the inhomogeneity while for B, C the source is located perpendicular to the plane of the figure. In A/a the reconstructed image would have uniform intensity so the inhomogeneity would appear transparent, thus contrasting sharply with conventional profiling results. For D/d the ghost image at intermediate depth reflects the smaller wave velocity of the object. The small single scatter object in E/e would probably be lost in a profiling survey.

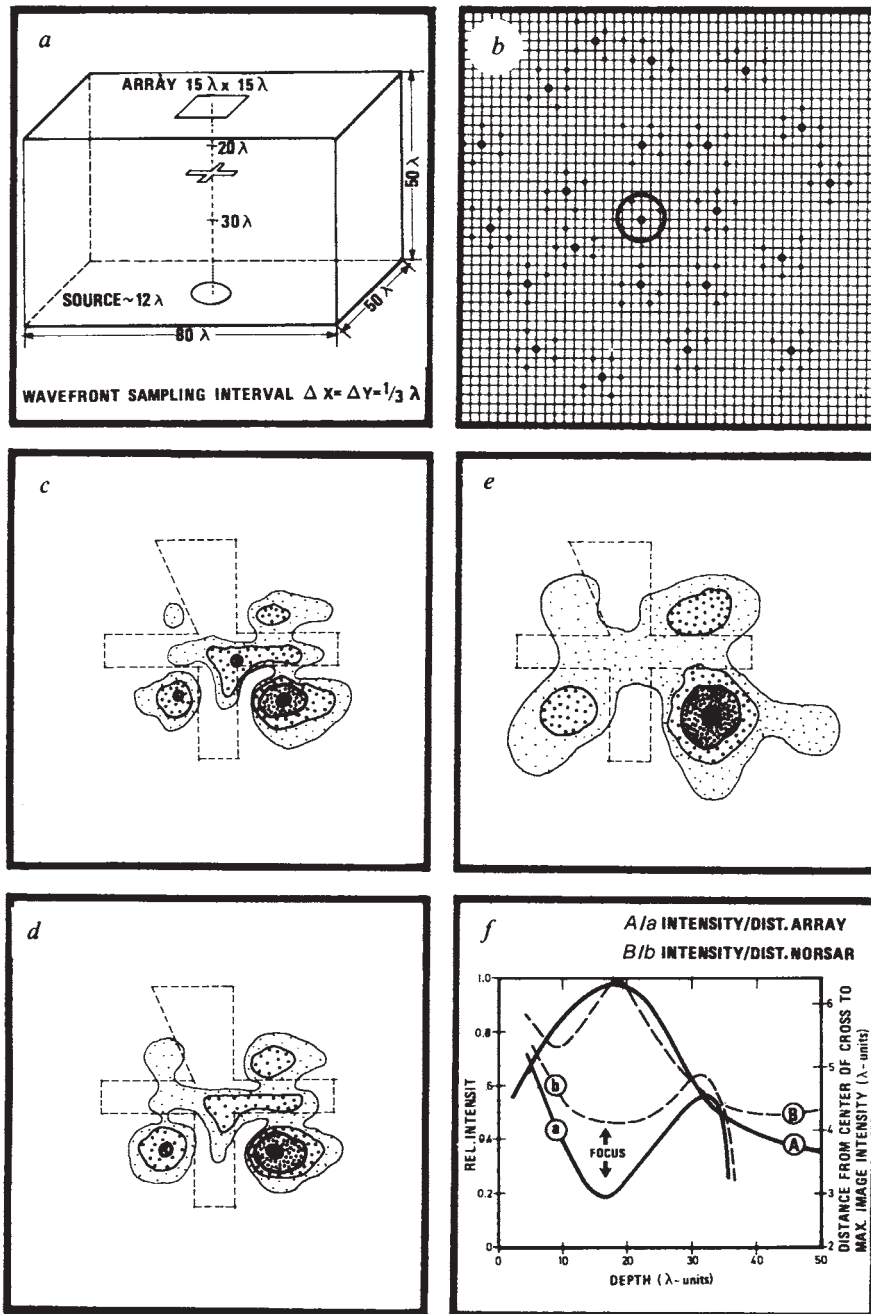


Fig. 2 Ultrasonic experimental set-up and results. *a*, The 'transparent' medium in the form of an epoxy box ($v_p = 2.6 \text{ km s}^{-1}$) and the embedded inhomogeneity in the form of an aluminium cross ($v_p = 5.2 \text{ km s}^{-1}$). The size of the box is $80 \times 50 \times 50 \lambda^3$ while the standard length and width of the individual arms in the cross are $\sim 12\lambda$ and $\sim 2\lambda$ respectively. *b*, The two sensor configurations used: (i) a rectangular grid with 2,025 sensors with an interspacing of $\lambda/3$ and (ii) a 132-sensor geometry similar to that of the NORSAR array. *c*, Image reconstruction of the cross (outlined) at its correct depth location on the basis of the regular grid network with contributions from all, 2,025 sensors to each reconstructed point. Relative intensity scaling is used with contour levels at 1, 2, 4 and 8 dB. *d* as *c* except that contributions are restricted to those observational points being within a radius equal to the diameter of the first Fresnel zone. The relatively high intensities observed at the centre of the cross in *c* and *d* stem from the non-plane source illumination. *e*, Image reconstruction of the cross when the observational points were limited to a simulated NORSAR array configuration of 132 sensors which in turn were extrapolated over a grid of 25×25 points with 0.6λ interspacing. As in *d* contributions to each reconstructed point were limited to a radius equal to the diameter of the first Fresnel zone. *f*, Relative intensity as a function of image reconstruction depth for both configurations of *b*. The strongest intensity is found at the proper depth location of the cross and this coincides with a minimum distance from centre of the reconstruction axis. The intensity estimations for depth exceeding $\sim 30\lambda$ reflects reconstruction of the source itself. The intensity maximum at the depth of the cross and the coinciding centre axis minimum distance both imply that depth resolution should be substantially better than that of three-dimensional time inversion^{4,5}.

indicated in Fig. 2a; the epoxy block dimensions would correspond to an upper mantle block of roughly $300 \times 300 \times 500 \text{ km}^3$. Wavefront amplitude $a(x, y)$ and phase $\phi(x, y)$ were recorded across the sensor aperture area in 2,025 sampling points. By recording phase information, the reference wavefield required in optics is superfluous. Also, using computerized image reconstructions the three-dimensional 'view' typical of optic holography is substituted by two-dimensional cross-sections at different levels. This experiment is also considered adequate for simulating the NORSAR sensor configuration (Fig. 2b).

The cross-image reconstruction results are displayed in Fig. 2c–e for the two sensor configurations shown in Fig. 2b, namely, the whole grid of 2,025 sensors (Fig. 2c, d) and that of simulating the 132 sensor positions of the NORSAR array (Fig. 2e). The cross-images in the upper left quadrants of Fig. 2c–e are not entirely satisfactory and this prompted a reexamination of the experimental design. It was discovered that the source-positioning was slightly out of focus (lower right quadrant) and the source radiation was not plane but of a complicated spherical form. Taking these deficiencies into account, enables the pecu-

liarities in Fig. 2c–e to be explained rationally. For example, the strongest intensity is found in the lower right quadrant reflecting the asymmetric positioning of the source itself. The non-plane source radiation effect gave rise to focusing effects in the range $16\text{--}23 \lambda$ below the array surface. This explains why the outer cross corners are not seen, and the existence of the centrally located intensity peak in Fig. 2c.

The results in Fig. 2c–e indicate that even in the case of gross holographic undersampling of the wave field, in particular for the simulated NORSAR configuration of 132 sensors only, a satisfactory reconstruction of the essential features of an embedded body seems feasible.

Another important aspect of the earth holography is that compared with other methods its depth resolution is probably very good. From optics the depth resolution would be 2λ and 1λ vertically and laterally for ideal cases. In the ultrasonic experiment the vertical depth resolution appears to be within 3λ (Fig. 3f) which is highly satisfactory. In practical earth holography the depth resolution is likely to be less impressive, say, within 5λ due to the relative complexities of lithospheric materials, but would still compare favourably with that obtained through

travel–amplitude inversion experiments. Other factors affecting the resolution in a rather complex way are array aperture, sensor spacing and depths to the anomalous bodies¹⁶.

Lithospheric heterogeneities

For testing the earth holography concept, NORSAR P-wave recordings of three deep earthquakes at teleseismic distances (South of Honshu, Hindu Kush and Western Brazil) were analysed. A characteristic feature of these recordings is that each of the 132 seismograms for one event exhibit an interference pattern of pulse-shaped waves propagating through the receiver region at slightly different paths. The first arrival is the direct (refracted) wave while scattering contributions arrive a little later. Obviously, the main information on lithospheric heterogeneities is confined to the initial part of the seismogram—here set to the first 10 s. The information extraction was tied to (spectral) amplitudes and phases for several harmonic components (0.6–2.6 Hz) of the recorded signals. However, as the 1.8-Hz component proved to be best, the results are restricted to this frequency. Note that the monochromatic signals of optical holography can be simulated by using a wideband frequency (or pulse) source in combination with sensors having resonant characteristics. The latter design is obtainable using digital filtering in time or frequency domain. Also, for image reconstruction the Earth's crust was given a thickness of 36 km and an average P-velocity of 6.5 km s^{-1} , while the lower part of the lithosphere was a half space with a P-velocity of 8.2 km s^{-1} . Because this analysis is extremely computer-time consuming and each additional event only provides an independent set of reconstructed images the analysis was restricted to three clear event recordings.

Results tied to image reconstruction of lithospheric heterogeneities under NORSAR using the south of Honshu earthquake recordings are given in Fig. 3, which shows only the most striking features. At a depth of 100 km (Fig. 3d) the prominent intensity maximum to the east was the most concentrated one found over 14 intervals in the depth range 0–250 km. This bright spot also has the closest location relative to the centre of the array (see Fig. 2 legend).

For the anomaly to the north a similar effect is attained at a depth of 148 km (Fig. 3b). As depth increases the two anomalies deteriorate while two weak anomalies near the centre of the array emerge (Fig. 3c). Composite results for all three events analysed are shown in Fig. 4 for the same depth intervals as used previously. Area I is a rather weak anomaly at a depth of 148 km which is not clearly 'seen' by the western Brazil event; area II is the brightest heterogeneity of shallow depth ($\sim 100 \text{ km}$) which gives energy maxima for all three events; area III—a rather strong inhomogeneity—is located near the centre of the array, and is most clearly seen in the western Brazil results. The three events indicate different depth locations for areas I, II and III (III being the deepest), and that suggests a significant vertical extent of the heterogeneous bodies. These are 'illuminated' differently by the respective events, for example, side scattering by a rather strong heterogeneity seems to be required for anomaly II to be seen by the Western Brazil event. For areas IV, V and VI the anomalies are rather weak and are seen most clearly by the Western Brazil event.

Discussion

Much research effort has been devoted to analysing the complex P-wave field observed across NORSAR, and both random medium and deterministic^{4,5} approaches have been utilized. The latter result, the essence of which is indicated in Fig. 4 in terms of relative P-velocity anomaly contours, is the most comprehensive obtained so far and is a natural choice for a comparison with the outcome of the holography experiment, although a one-to-one overlap between the inversion results cannot be expected; time inversion has relatively poor depth resolution whereas earth holography results depend partly on the direction of the wave approach. Nevertheless, the relatively marked image intensities of areas II and III coincide reasonably well with the strong

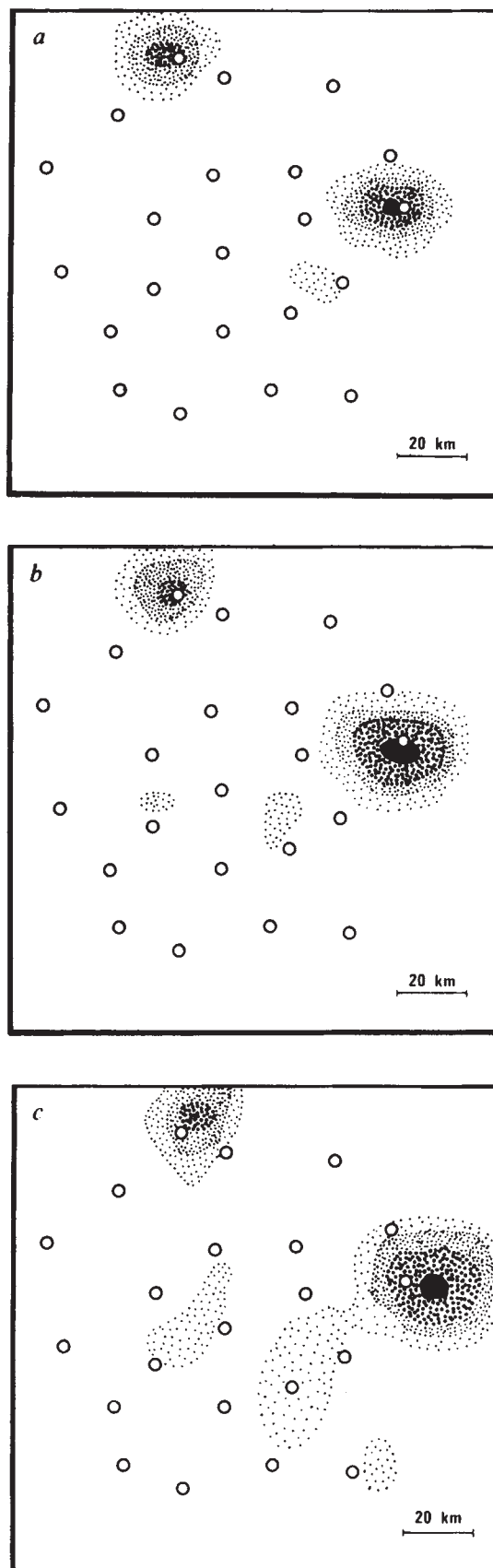


Fig. 3 Image reconstructions at depths of: *a*, 100 km, maximum intensity = 0 dB; *b*, 148 km, maximum intensity = -1.8 dB; and *c*, 212 km maximum intensity = -7.5 dB for the south of Honshu event using the computational procedure as indicated in Fig. 2e legend, frequency = 1.8 Hz. The intensity grading in each figure is in steps of 1 (black), 2, 4 and 8 dB down from maximum. The strongest intensity is to the east in *a* and is also the most concentrated. Note that this intensity has the closest location *vis-à-vis* the array centre and in this respect is similar to the focusing effect in Fig. 2f.

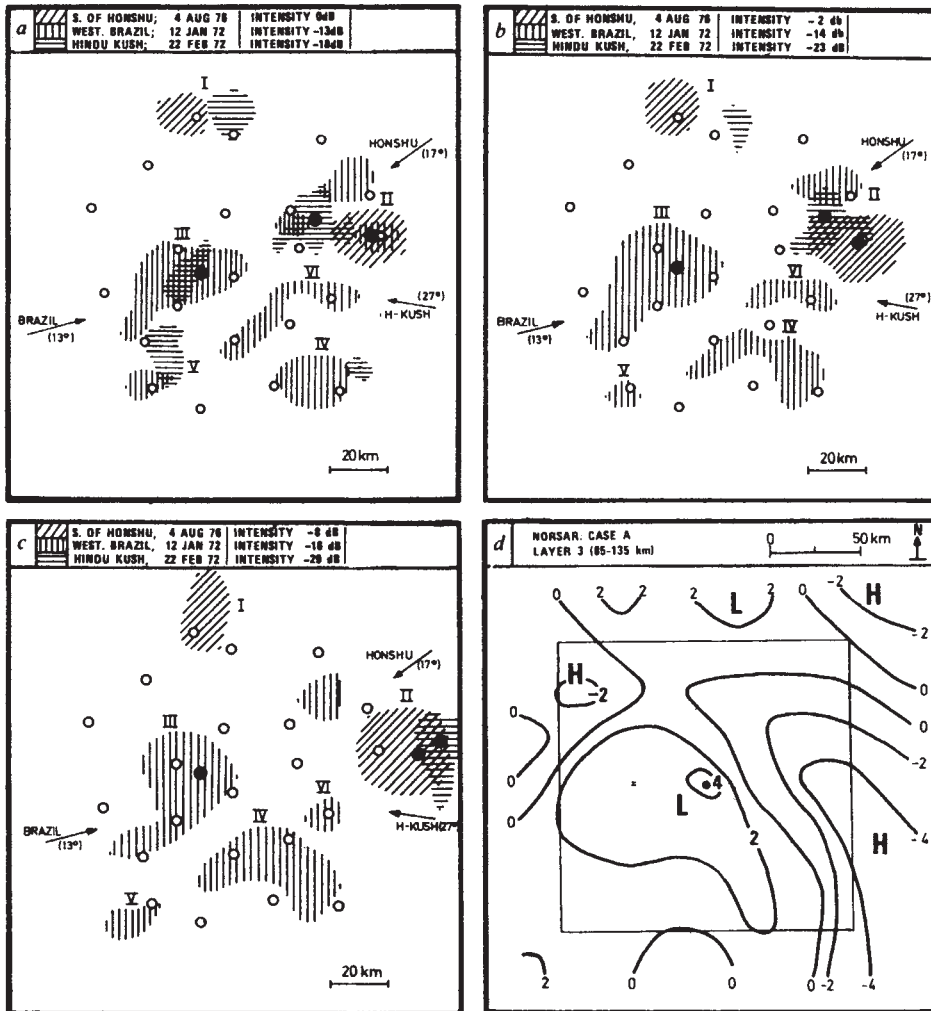


Fig. 4 Composite display of intensity maxima for depths: *a*, 100 km; *b*, 148 km; and *c*, 212 km exhibiting the most pronounced scattering features, frequency = 1.8 Hz. Different shading is used to identify the three events and their relative intensity scaling is also given. The contours of the various shaded areas are 4 dB down from the individual event maxima. A good overlap in *a* between the intensity areas for the different events implies that heterogeneities at this depth are most uniformly seen by the three events. *b*, Which has weaker intensities than *a*, also exhibits less of an overlap between the respective event intensity areas. Similar comments apply to *c*. The consistency in location of the intensity areas I, II and III between *a*, *b* and *c* implies a significant depth extent of the heterogeneous bodies. For comparison the P-residual inversion results⁵ in terms of relative velocity perturbations in per cent for model A of ref. 5, depth interval 85–135 km are displayed in *d*. There is reasonable agreement between these two types of seismic results. Note that the time inversion results for the depth interval 185–235 km are essentially the same as those for the 85–135 km interval, implying decreasing resolution with increasing depth with this method.

velocity anomalies obtained by direct inversion of travel time residuals. The intermediate image intensity of area I does not have a clear counterpart in the residual inversion results, and the same applies to the weaker area VI. In both these cases pronounced velocity changes occur nearby. The weak area V, may actually belong to area III as both are within the pronounced velocity low in the southwestern quadrant. The area IV inhomogeneity which is also weak may be associated like area II with the velocity high to the east. Area VI which is seen only by the Western Brazil event may be very deeply located (say > 200 km) and by that poorly resolved in view of the array's aperture of only 100 km.

We now consider the results of Fig. 4 in terms of geological structures. The fact that none of the anomalous areas observed seem to originate at shallow depth (≤ 80 km) is not unexpected as, with NORSAR's rather coarse sampling, the crust would appear relatively transparent. Thus, no obvious correlation was found with surface geological features such as the Oslo graben nor with a mylonite to the south-east. Except for the seismic manifestation of the Oslo graben⁵ weak impedance contrast this agrees well with time residual inversion results. The anomalous body corresponding to the imagery reconstructions of areas I, II and III seems to represent bodies of different impedance contrasts and also different vertical extent. However, from the implied vertical extent of the heterogeneities corresponding to areas I, II and III we conclude that predominantly vertical processes take place within the lithosphere¹⁷ and in particular in association with taphrogenesis¹⁸.

The interpretation of the results displayed in Figs 3 and 4 depends on the depth resolution of the associated anomalous areas which was discussed above with basis in optics. In earth holography the conditions are more difficult with noise in the

observations, the probable vertical extent of the anomalies and the general complexities of lithospheric structure. However, our previous estimate of 5λ in the vertical direction seems to be confirmed by the intensity depth sharpness of the anomalous areas I, II and III and in this respect is substantially better than that obtained in the inversion experiments. For example, the Fig. 4f time inversion anomalies for the depth interval 85–135 km remain essentially unchanged when projected 100 km lower down⁵. The same applies to amplitude inversion experiments⁷.

Conclusions

We have demonstrated that acoustical holography can be applied to seismological observations from a large aperture array such as NORSAR. The results obtained in terms of located heterogeneous bodies in the lithosphere are in reasonable agreement with inversion results based on time residual observations from 183 events even though only three events were used for this analysis. Also, the depth resolution of our earth holography approach seems to be much better than that of the inversion technique and is estimated to be within 5λ or 20 km in this particular case. The earth holography technique uses deterministic scattering effects often ignored in traditional seismic investigations and thus provides important, supplementary information on lithospheric heterogeneities.

The earth holography technique could be used on scales comparable with those used in seismic prospecting say for locating ore bodies, fault mapping and other sources of strong diffracted waves. However, the necessity of sufficiently dense sampling restricts its practical usage at present but its future application should be possible with improvements in digital recording techniques.

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Base pairing of RNA I with its complementary sequence in the primer precursor inhibits ColE1 replication

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Four single base pair mutations in the region that codes for RNA I create new incompatibility groups while preserving the mechanism of control of initiation of DNA replication in a ColE1-type plasmid. Sequence analysis of these base substitutions points to the primary importance of the central loop of the cloverleaf structure of RNA I in this control mechanism.

PLASMIDS offer an attractive model for investigating the molecular mechanisms that ensure controlled replication of bacterial replicons during each division cycle. In many plasmids two species of RNA molecules, coded for in the region of the replication origin, seem to have a crucial role in the control of plasmid replication^{1–6}. One of these, RNA II, is transcribed from a point upstream from, and towards, the replication origin, terminating at a point beyond it. In ColE1-like plasmids RNA II molecules have been shown *in vitro* to be processed at the origin of replication by RNase H (ref. 3) to form primers for the initiation of DNA synthesis by DNA polymerase I. The second, and smaller, RNA species, RNA I, is transcribed in the opposite direction to RNA II from the region of the plasmid genome shown genetically to determine plasmid copy number^{1,2,5}. *In vitro*, RNA I molecules prevent the maturation of RNA II molecules, in a way that is not understood⁶. RNA I only inhibits primer formation by the plasmid that codes for it, having no effect on heterologous plasmids⁶, and it has been proposed that this interaction controls plasmid copy number. Here we show that single base pair substitutions in the region complementary to the central loop of RNA I's putative cloverleaf structure create new compatibility groups (defined by replication of the mutant in bacteria harbouring plasmids with homologous replication origins) by changing the specificity of the target of RNA I and suggest a model for the inhibitory interaction.

Isolation of target mutants

We expected that mutants altered in the target of the inhibitor of plasmid replication would be dominant high copy number mutants. However, rather than using direct selection for plasmids with a higher number of copies, we used an approach involving the phasmid system⁷, so as to avoid overlooking mutant plasmids potentially lethal for the host and to select against recessive mutants. This approach is particularly suitable for genetic analysis and relies on the possibility of incapsidating plasmid sequences in a λ phage head (Fig. 1).

We have previously observed⁷ that the insertion of the plasmid *pac129* (Fig. 3) into the λ chromosome by *int*-mediated recombination confers on the hybrid the ability to grow on a homoimmune lysogen. This virulence is due to titration of λ

repressor by the extra copies of λ operators that result from replication of the hybrid using the plasmid origin. The presence in the lysogenic strain of a resident plasmid that synthesizes the inhibitor of plasmid replication prevents virulence by blocking the initiation of plasmid DNA synthesis in the incoming phasmid. A phasmid with an altered inhibitor target will be able to escape repression and will be virulent even in the presence of a resident plasmid (Fig. 1a).

The isolation of such inhibition-insensitive mutants (*svir*) will be described in detail elsewhere⁸; here we summarize their properties. Six independent *svir* mutants were isolated. Only four of them can be released efficiently from the λ chromosome by infection of an *int* constitutive strain (Fig. 1b); the remaining two mutants, *svir3* and *svir19*, are lethal for the host and cannot be propagated as plasmids. Three of the mutant plasmids that replicate efficiently show an increase in relative copy number with respect to wild type (Table 1). This increase is dominant in a complementation test. Unexpectedly, the remaining mutant, *svir11*, is present in the host bacterium with a lower number of copies than the wild type (0.7 relative copy number), as if the mutation had created a better mechanism of self repression. From the behaviour of these mutants in compatibility experiments we conclude that the target of the inhibitor of ColE1-type replication coincides with part of the inhibitor coding sequence⁸. As a consequence, single mutations in the target result in altered inhibitors that are unable to interact with the wild-type target. This hypothesis is consistent with what is known about the transcriptional organization of the replication region of ColE1 and the finding that RNA I inhibits primer formation by acting somewhere further upstream than 300 nucleotides from the origin⁶.

Specificity test

If *svir* plasmids are mutated not only in the target but also in the repressor, we are left with the puzzle of the low copy number of *svir11*. One possible explanation would be that a single base pair change had modified target and repressor in a complementary way that maintained and possibly improved the interaction between the two mutated elements. To test this hypothesis we devised the test shown in Fig. 2 where each *svir* plasmid

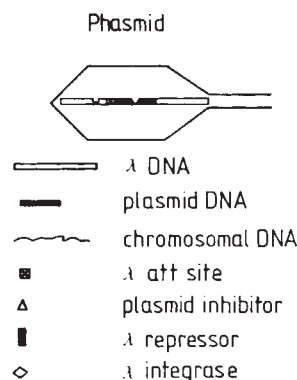
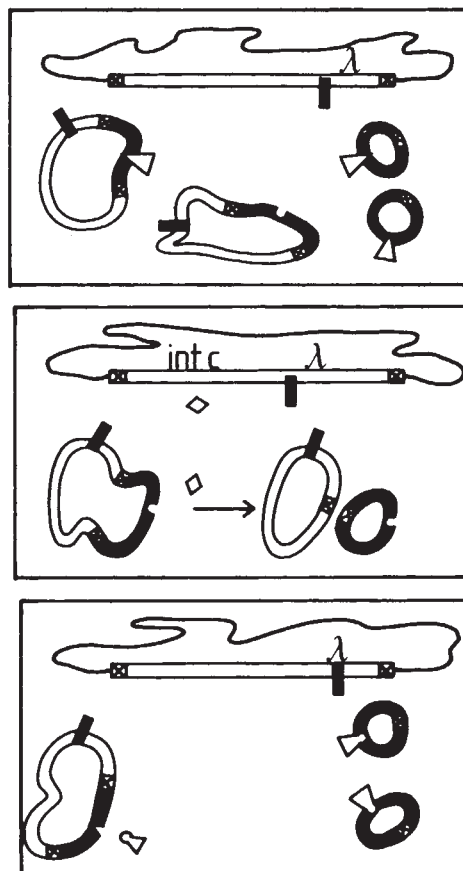


Fig. 1 The phasmid system. The drawings illustrate schematically how a phasmid can be used to select dominant mutants in the target of the negative regulator of plasmid replication. The phasmid⁷ is a hybrid obtained by *in vivo* integration into the λ chromosome of a plasmid containing an *att* site. The resulting hybrid molecule can be encapsulated into λ heads and, as a consequence, plasmid sequences can be injected into different bacterial environments by phage infection. Infection of a lysogen containing a plasmid of the same compatibility group (a) will not lead to any phage production. Both phage and plasmid replication system are, in fact, blocked. Phasmid mutants carrying an altered target for the inhibitor of plasmid replication can replicate under plasmid control and start a lytic cycle when the copy number reaches a value sufficient to titrate-repressor. The process of plasmid integration is reversible and plasmid sequences can be excised from the λ chromosome (b) by infecting the strain EQ84 = *HfrH galT* $\Delta(int-FII)$ (λ *intc*226) that constitutively synthesizes λ integrase. Clones that contain the original plasmid sequences can be selected at 30 °C on plates containing 50 μ g ml⁻¹ of ampicillin. The experimental details of the specificity test (c) are described in Fig. 2 legend.



(horizontal lines) was spotted on a lawn containing a permissive indicator together with a lysogenic strain harbouring the different *svir* plasmids (vertical lines). A turbid spot indicates that the phasmid is unable to replicate in the corresponding lysogenic strain. Insensitivity to the inhibitor synthesized by the resident plasmid will result in phasmid replication, virulence and, as a consequence, in a clear spot. Experiments of the type shown in Fig. 2 clearly indicate that not only *svir11*, but also the three high copy number *svir* mutants, can still synthesize an active inhibitor. Furthermore, the turbid spots are present only in the diagonal, indicating that the mutant inhibitors are active only on their own target and not on the targets of the wild-type plasmid or of the remaining mutants. The only exceptions are *svir2* and *svir7*, which seem to have the same specificity. Our sequence data identify them as identical.

We conclude from these experiments that a mutation in the target of this repression system causes a complementary alteration of the inhibitor itself so that the control mechanism remains functional despite the change in specificity. These properties are consistent with a repression mechanism based on base pairing between complementary nucleic acid.

svir mutants define new compatibility groups

The 'inhibitor dilution' model⁹ proposes that incompatibility is the result of the inhibitory activity of a negative regulator of plasmid replication that is unable to distinguish between two homologous replicons. Given the results of the specificity test in Fig. 2, this model predicts that each *svir* mutant should be compatible with all the others but incompatible with itself. To test this prediction we constructed (Fig. 3) tetracycline-resistant derivatives of the wild-type plasmid and of the three mutants with different specificities (*svir7*, *svir11*, *svir12*). Heterozygous cells containing all the possible combinations of ampicillin- and tetracycline-resistant derivatives were constructed by co-transformation of the *recA* strain HB101. The percentage of clones

Plasmid present in the lysogen None Pace29 *svir2* *svir7* *svir11* *svir12*

Infecting phasmid
vir⁻ 100
 Φ 1 WT
 Φ *svir2*
 Φ *svir7*
 Φ *svir11*
 Φ *svir12*

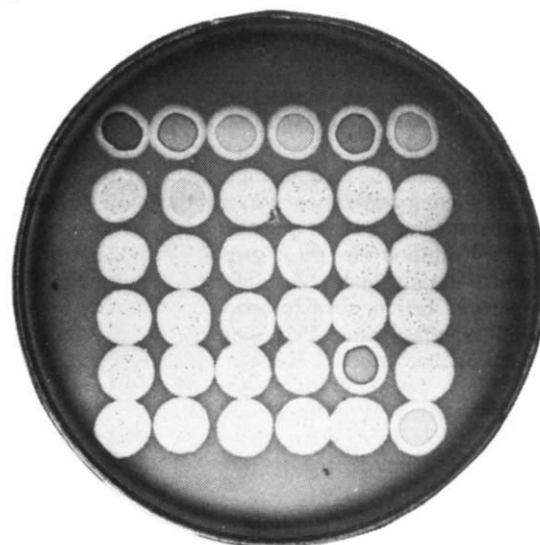


Fig. 2 Specificity test. The plate was prepared by overlaying a BBL plate (Baltimore Biological Laboratories; trypticase 10 g, NaCl 5 g, Difco agar 10 g l⁻¹) with 3 ml of top agar (as BBL agar but only 6.5 g agar per l) containing 0.2 ml of a saturated culture of GC57 = $\Delta(lac-pro)malA$ *metB* *argE*_{am} *rif* *thi* *supF*. This strain is used as a permissive strain for all the phasmids tested. 0.3 ml of a dilution of phasmid lysates containing $\sim 10^7$ plaque-forming units per ml was added to the wells of the horizontal lines of a microtitre plate. The wells in the vertical lines of the resulting grid were further inoculated with 0.05 ml of the lysogenic strain Q37 (*thr* *leu* *lacY* *supE* (λ P_{am3})) containing the plasmid *pac129* or one of its *svir* derivatives. 5 μ l of the content of each well were spotted in a corresponding grid on the plate containing GC57. The photograph was taken after 24 h incubation at 37 °C. *vir100* is a replication-defective derivative of the phasmid Φ 1 (ref. 8). WT, wild type.

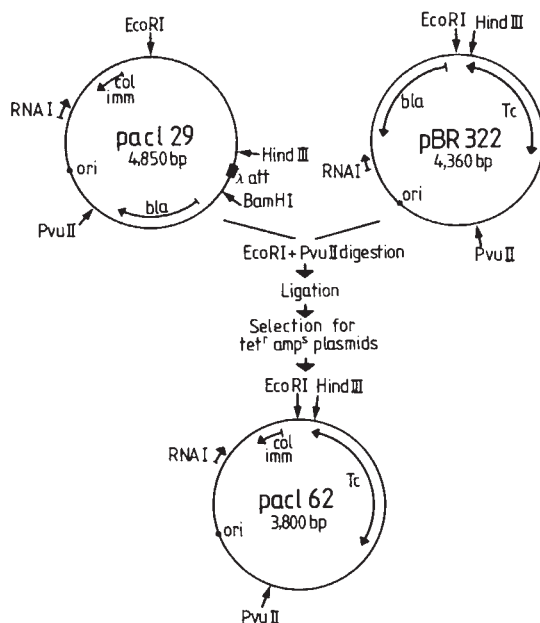


Fig. 3 Incompatibility properties of *svir* derivatives. Tetracycline-resistant derivatives of the plasmid *pac129* and of the three *svir* mutants with different specificities, were constructed using the strategy shown. DNA from the plasmid *pBR322*¹⁴ and *pac129* was digested with the restriction endonucleases *EcoRI* and *PvuII*. Equimolar amounts of the two digested DNAs were mixed at a concentration of $10 \mu\text{g ml}^{-1}$ and ligated overnight at 14°C . After transformation of a culture of HB101 made competent by CaCl_2 treatment, the transformed clones were selected on tetracycline plates ($10 \mu\text{g ml}^{-1}$). The desired *pac162* structure can be recognized from reconstituted *pBR322* by replica plating on ampicillin plates ($100 \mu\text{g ml}^{-1}$). *svir7* and *svir12* *pac162* derivatives have lost the *PvuII* site in this construction probably because of some exonuclease activity in the enzyme preparation.

that had lost either of the two plasmids after growth in non-selective medium was monitored approximately every 20 generations by plating in the presence of antibiotics. Table 1 shows the number of generations required to decrease by one order of magnitude (D_{-1}) the number of clones containing both plasmids. As predicted by the inhibitor dilution model, only combinations that have cross-reacting inhibitors result in rapid segregation of clones containing only one of the two plasmids. After 150 generations, less than 1 out of 200 clones had lost one of the two plasmids in the segregation experiments on either side of the diagonal of Table 1.

These compatibility experiments support the hypothesis raised in the previous section and prove that, as predicted by the inhibitor dilution model, an alteration of the specificity of the inhibitor–target interaction results in altered compatibility properties. Given the peculiar repression system devised by ColE1-type replicons, it is possible to create new compatibility

Table 1 D_{-1} values in segregation experiments

Relative copy number	Ampicillin-resistant plasmid	Tetracycline-resistant plasmid			
		<i>pac162</i>	<i>svir7</i>	<i>svir11</i>	<i>svir12</i>
1	<i>pac129</i>	40–50	>150	>150	>150
3.5	<i>svir7</i>	>150	40–50	>150	>150
0.7	<i>svir11</i>	>150	>150	60–70	>150
1.5	<i>svir12</i>	>150	>150	>150	50–60

D_{-1} values are given for the segregation experiment of bacterial strains containing the various combinations of ampicillin- and tetracycline-resistant derivatives. Clones containing two plasmids were constructed by co-transformation and selection on plates containing both antibiotics. One doubly resistant clone for each transformation was plated on an L plate and one colony still resistant to both antibiotics was grown for several generations (with dilutions in the plateau phase) in non-selective conditions. Every 20 generations the cultures were titrated and one plate containing ~200 colonies was replica-plated on to ampicillin and tetracycline plates. The number of generations required to decrease by a factor of 10 the percentage of cells containing both plasmids (D_{-1} values) were obtained from such segregation curves. The values in the diagonal are the average of three independent determinations. The first column shows the relative copy numbers of *svir* mutants⁸.

groups by single base pair changes. Our results are not consistent with models of the type proposed by Bedbrook *et al.*¹⁰ that stress the importance of membrane replication–segregation sites in determining plasmid incompatibility.

The D_{-1} values of the different *svir* mutants were not proportional to plasmid copy number. We cannot explain this result, which seems to conflict with models based on random replication and random segregation^{11,12}.

Location of *svir* mutants

The four base pair changes that cause the change in specificity of the control mechanism have been previously⁸ localized by *in vivo* deletion mapping between nucleotides 295 and 511 upstream from the origin of plasmid replication. This DNA fragment was sequenced by the chemical degradation method¹³ and revealed in each case a single base pair transition in the region that codes for RNA I. Figure 4 shows the base changes

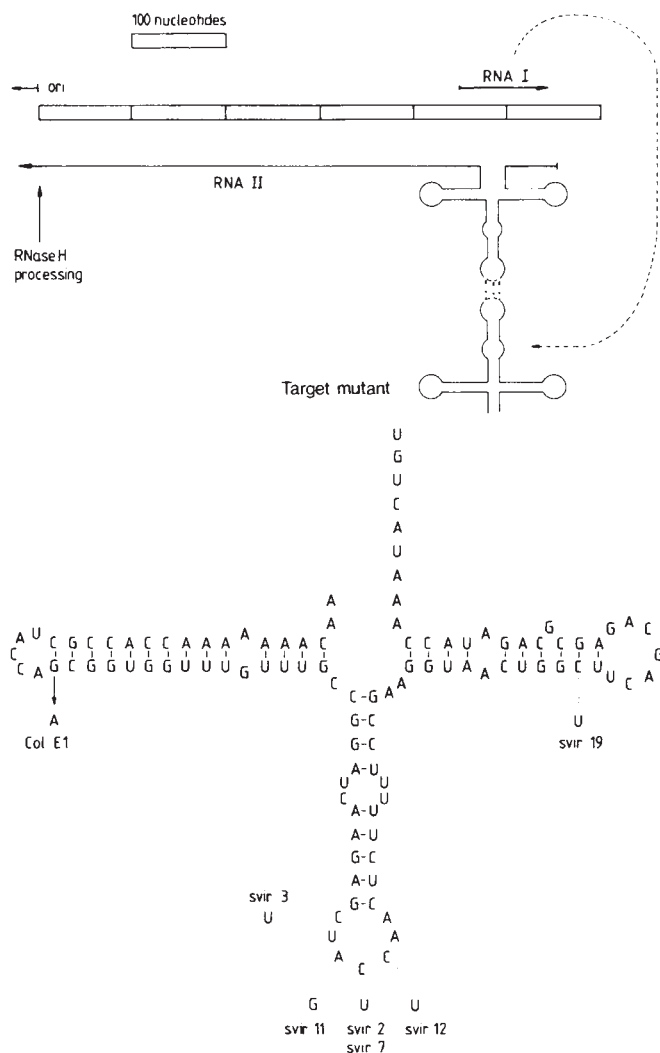


Fig. 4 Model of the inhibitory interaction between RNA I and the primer precursor and DNA sequence of the mutants. The DNA sequence of the wild-type *pac129* (no difference from the published *pBR322* sequence¹³) and of the *svir* mutants was obtained using the chemical degradation method¹³ after 3' end labelling with DNA polymerase (Klenow subunit). The wild type and the two mutants obtained with nitrosoguanidine mutagenesis (*svir2*, *svir7*) were sequenced from the *PvuII* site ~500 nucleotides downstream from the origin of replication up to the end of RNA I in order to be able to exclude the possibility of secondary mutations. *svir11* and *svir12* (obtained by *mutD* mutagenesis) were sequenced only in the *HpaII* fragment that was shown by deletion mapping to contain the mutation. *svir3* and *svir19* cannot be propagated in the plasmid form. The *Sau3A*–*TaqI* DNA fragment that was used for sequencing was therefore prepared from phasmid DNA. The arrow in the right loop of the RNA I cloverleaf structure points to the unique base difference in the RNA I synthesized by the related plasmid ColE1. The *svir* mutations are shown as alterations of the RNA sequence of the primer precursor.

with respect to the wild-type sequence. The three base transitions at positions 55, 56 and 57 of the RNA I sequence indicate the importance of this triplet in the nucleic acid-nucleic acid interaction controlling pMB1 replication. The number of mutations isolated and sequenced is insufficient to define the limits of the site of interaction between repressor and target. Nevertheless, the clustering of the four mutations at the top of the central loop of the cloverleaf structure of RNA I is reminiscent of a 'codon-anticodon type' interaction between transfer RNA and messenger RNA. We do not know whether this observation has any functional or evolutionary significance.

Target mutants that alter the cloverleaf structure

The two remaining inhibitor-insensitive mutants, *svir3* and *svir19*, when released from the λ chromosome, are lethal for the host cell. We assume that this phenotype is due to uncontrolled replication. Strong selection for plasmid release and maintenance results in deletions of part of the mutant plasmid. The two mutations were localized by deletion mapping between nucleotides 295 and 511 from the origin of DNA replication. The corresponding DNA fragment was prepared from phasmid DNA and sequenced by the chemical degradation method. *svir3* was found to be a base substitution that might affect target function by extending the central stem of the cloverleaf structure, thus preventing the anticodon-type base pairing with the inhibitor. The tRNA-like secondary structure is also disturbed by the C $\rightarrow$ T transition in *svir19* that affects the stability of one of its stems.

Conclusions

In four of the *svir* mutants that have been characterized, single base pair transitions resulted in a change of specificity in the mechanism of control of copy number without destroying its function. We believe this to be the first description of such a mechanism, which allows evolution while ensuring protection of function. It is possible that the relevance of this mechanism extends to biological phenomena in which self recognition plays

a role. A single mutation may allow for the evolution of a new class of specificity while preserving, through the complementary alteration of the target sequence, the mechanism of self recognition.

Our results point to the crucial function of the central loop of RNA I in the interaction that leads to inhibition of initiation of ColE1 replication. Conclusions about the bases involved in these interactions must await the isolation and characterization of more mutants of different specificities. The specificity test that we have devised (Fig. 2) offers an easy tool for this type of approach.

It is interesting that the mutant copy number phenotype is correlated with the free energy content of the hydrogen bonds between the nucleotides of the two triplets postulated to be involved in the interaction. C $\rightarrow$ U transitions respond to increase in copy number while the only A $\rightarrow$ G transition results in a low copy number plasmid, probably as a consequence of the better repressor target interaction. The dramatic change in the efficiency and specificity of the control mechanism caused by single base substitutions suggests that this interaction does not involve a large number of bases.

From our results and the *in vitro* experiments of Tomizawa *et al.*⁶, we propose (Fig. 4) that the base pairing between the central loop of RNA I and the complementary structure on the nascent primer precursor is essential in preventing maturation of this latter transcript, probably by preventing the formation of the RNA-DNA hybrid that is the substrate for the maturation enzyme RNase H. Furthermore, we have evidence that the stability of the cloverleaf structure of the target is a requirement for successful inhibitory interaction. Two mutations that alter two of the stems in the target putative secondary structure result in insensitivity to wild-type inhibitor. Our results, however, do not allow us to distinguish whether RNA I interacts with the primer precursor or with its own DNA coding sequence. This will require further genetic and biochemical experiments.

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Two conserved sequence blocks within eukaryotic tRNA genes are major promoter elements

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The split promoter sequences of a tRNA^{Leu}_{CUG} gene of Xenopus laevis have been mapped to nucleotides 13-20 and 51-64 of the tRNA^{Leu} coding sequences. The sequences closely coincide with two conserved sequence blocks present in all eukaryotic tRNA genes. The two conserved sequence blocks were found to be exchangeable between tRNA genes as chimaeric tRNA^{Met}-tRNA^{Leu} genes proved transcriptionally active. Furthermore, two prokaryotic tRNA genes exhibiting strong homologies with the two blocks yielded specific transcripts when tested in an eukaryotic transcriptional system.

THE tRNA^{Met}_{AUG} genes of *Xenopus laevis* have an unusual split-promoter arrangement in that sequences directing transcription are located near both the 5' and the 3' end of the tRNA^{Met} gene unit¹. As in the case of the 5S RNA gene^{2,3} of the same genus

and the VAI gene of adenovirus⁴, the control regions of the tRNA^{Met} are located within the structural gene itself (reviewed in ref. 5). Here, we show that a tRNA^{Leu}_{CUG} gene of *X. laevis* has a similar split promoter and trace the essential sequences to two

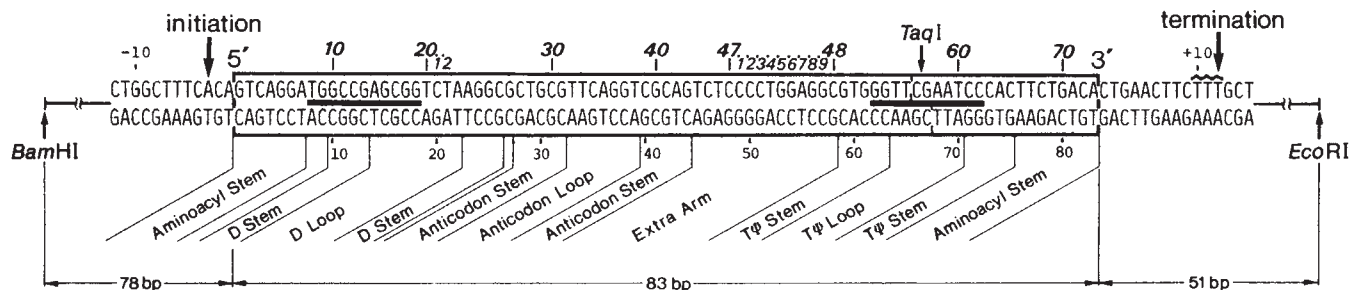


Fig. 1 DNA sequence of the tRNA^{Leu} gene of *X. laevis*. The DNA sequence coding for the tRNA^{Leu} is boxed. The numbers in bold-faced type above the box are in agreement with the standardized numbering system⁷. Consecutive numbering is shown below the box. The bars between the two DNA strands show the location of the conserved elements which are discussed in detail in Fig. 4 legend.

sequence blocks which are highly conserved in all eukaryotic tRNAs. We present evidence that the promoter organization of genes transcribed by polymerase III can differ substantially and argue that the split promoter arrangement of the tRNA genes is in keeping with the evolution and functional diversity of the tRNA structure.

The tRNA^{Leu} gene transcription unit

Figure 1 displays the DNA sequence of the *X. laevis* tRNA^{Leu} gene⁶ used in the present study, together with the DNA sequences of the adjacent flanking regions. The numbers in bold-faced type above the DNA sequence of the structural gene are in accord with the standard tRNA numbering system⁷, which is based on the primary structure of the yeast tRNA^{Phe} and is used here throughout. The tDNA^{Leu} fragment was isolated from a cloned 3.18-kilobase (kb) DNA repeat⁸ by cleavage with *Mbo*I and *Eco*RI and was then cloned between the *Eco*RI and *Bam*HI sites of pBR322. On injection of the recombinant plasmid with [α -³²P]GTP into the nuclei of frog (*Xenopus laevis*) oocytes⁹ (for technical details see ref. 10) followed by a short incubation period of 3 h, three labelled RNAs (m, p₁ and p₂) were detected by electrophoresis on denaturing polyacrylamide gels (Fig. 2, lanes a, b). Band m was previously identified by RNA sequencing as mature tRNA^{Leu} (S. Clarkson and R. Koski, personal communication) containing 85 bases, including the common 3'-terminal CCA sequence which is added post-transcriptionally. The two larger RNAs p₁ and p₂ are 98 and 95 nucleotides long, respectively, and represent precursors to the mature tRNA^{Leu}. The intensity of band m increased with longer incubation times whereas bands p₁ and p₂ were only faintly visible after overnight incubation.

The approximate position of the 5' termini of p₁ and p₂ on the tDNA was determined by S₁ mapping experiments^{5,11} using individual, gel-purified p₁ and p₂ RNA and a complementary hybridization probe, 5'-end labelled at the *Taq*I site (see Fig. 1). After S₁ digestion of the hybrids, the products were fractionated on a denaturing polyacrylamide gel: p₂ and m gave identical S₁-protected DNA bands (Fig. 2, lanes f, g) and hence these molecules are presumed to have identical 5' termini; therefore, both molecules have a G as the first base in the sequence. The DNA fragments protected by p₁ were two or three bases longer, hence the 5' end of p₁ maps at the A or C at position -3 or -2. We next investigated whether both p₁ and p₂ could be primary transcripts. [β -³²P]GTP or -ATP were injected with the tRNA^{Leu} gene and RNA was isolated after a short 30-min incubation to avoid transfer of the label to the α position⁵. Injection with [β -³²P]GTP did not result in labelling of any of the transcripts, whereas injection of [β -³²P]ATP labelled only the p₁ transcript (Fig. 2i). We conclude that p₁ is a primary transcript commencing with an A at position -3 and that p₂ and m are derived from p₁ by processing at the 5' end. Considering the length of p₁ and p₂, it seems likely that the 3' end of both precursors maps to position +11 or +12, that is, to the two last bases of the putative termination signal TTCTTT in the 3'-flanking sequence.

The intragenic promoter

Having established the size of the tRNA^{Leu} transcription unit, we constructed deletion mutants lacking sequences at either the 5' or the 3' end of the tRNA^{Leu} gene. The *Bam*HI and *Eco*RI sites at positions -78 and +51, respectively, provided convenient starting points for resection with exonuclease *Bal*31. After the addition of *Hind*III linkers to the resected ends, the truncated tDNA^{Leu} fragments were recloned, all in the same orientation, into the pBR322 vector, between either the *Eco*RI and *Hind*III or the *Bam*HI and *Hind*III sites. The extent of the sequence truncation was determined by DNA sequencing¹² and its effects on transcription finally assessed by oocyte injection. To normalize the amount of transcription in each injection experiment, cloned 5S DNA of *X. laevis*¹³ was routinely co-injected as an internal control at a level of one 5S RNA gene per four tRNA^{Leu} genes.

The resected clone *a* (Fig. 3), which lacks most of the 5'-flanking region but still contains the precursor transcription unit, is transcribed at the same level as the progenitor clone. Further

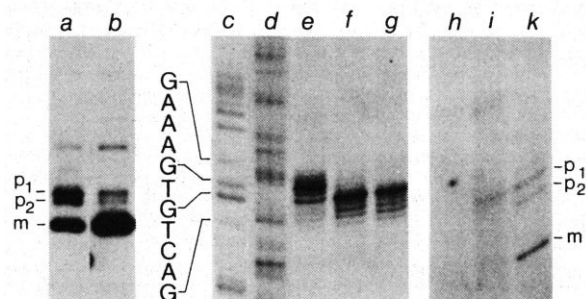


Fig. 2 Analysis of transcripts made from the tRNA^{Leu} wild-type gene. The small DNA fragment shown in Fig. 1 was isolated from a cloned 3.18-kb DNA repeat unit⁸ after cleavage with *Eco*RI and *Sau*3A and was then cloned with pBR322 between the *Eco*RI and the *Bam*HI restriction sites. Plasmid DNA was isolated by the clear-lysate method²² and further purified by isopropanol precipitation and centrifugation in a CsCl step gradient. After recovery and reprecipitation, the DNA used for oocyte injection was dissolved in 88 mM NaCl, 10 mM Tris HCl pH 7.4, at a concentration of 200 μ g ml⁻¹. A 20 nl aliquot of this DNA solution containing 0.2 μ Ci of [α -³²P]- or 0.4 μ Ci of [β -³²P]-triphosphates was injected into nuclei of centrifuged oocytes as described earlier^{9,10}. The oocytes were incubated for 0.5–16 h and total nucleic acids then isolated. The labelled RNAs were analysed by electrophoresis through 10% polyacrylamide, 8 M urea sequencing gels and detected by exposure to X-ray film using an intensifier screen. For S₁ mapping, individual RNAs (m, p₁ and p₂) were isolated from the gel¹² and the RNA from one oocyte was hybridized to the 5' half of a tDNA^{Leu} fragment which had been 5'-end labelled at the *Taq*I site (compare Fig. 1). Hybridization was carried out in 20 μ l of 80% formamide, 0.4 M NaCl, 40 mM MOPS pH 6.7, and 1 mM EDTA at 51 °C for 16–24 h. The reaction was stopped on ice with 180 μ l ice-cold S₁ buffer (30 mM NaAC pH 4.5, 2 mM ZnSO₄, 0.2 M NaCl). 10 U of S₁ enzyme were added and the samples were incubated at 41 °C for 45 min. The analysis of the protected DNA fragments was carried out on a 10% polyacrylamide, 7 M urea sequencing gel. Lanes a, b, RNA isolated from two oocytes incubated for 3 (a) and 15 (b) h; lanes c, f, g, S₁ mapping of p₁ (e), p₂ (f) and m (g); lanes c, d, A-specific (d) and G-specific (c) sequence reaction of the DNA probe used for S₁ mapping; lanes h, i and k, labelling with [β -³²P]GTP (h) and [β -³²P]ATP (i) and [α -³²P]GTP (k) for 0.5 h. RNAs extracted from 46 (h), 52 (i) and 1.5 (k) oocytes were loaded onto the gel. Exposure time was 48 h.

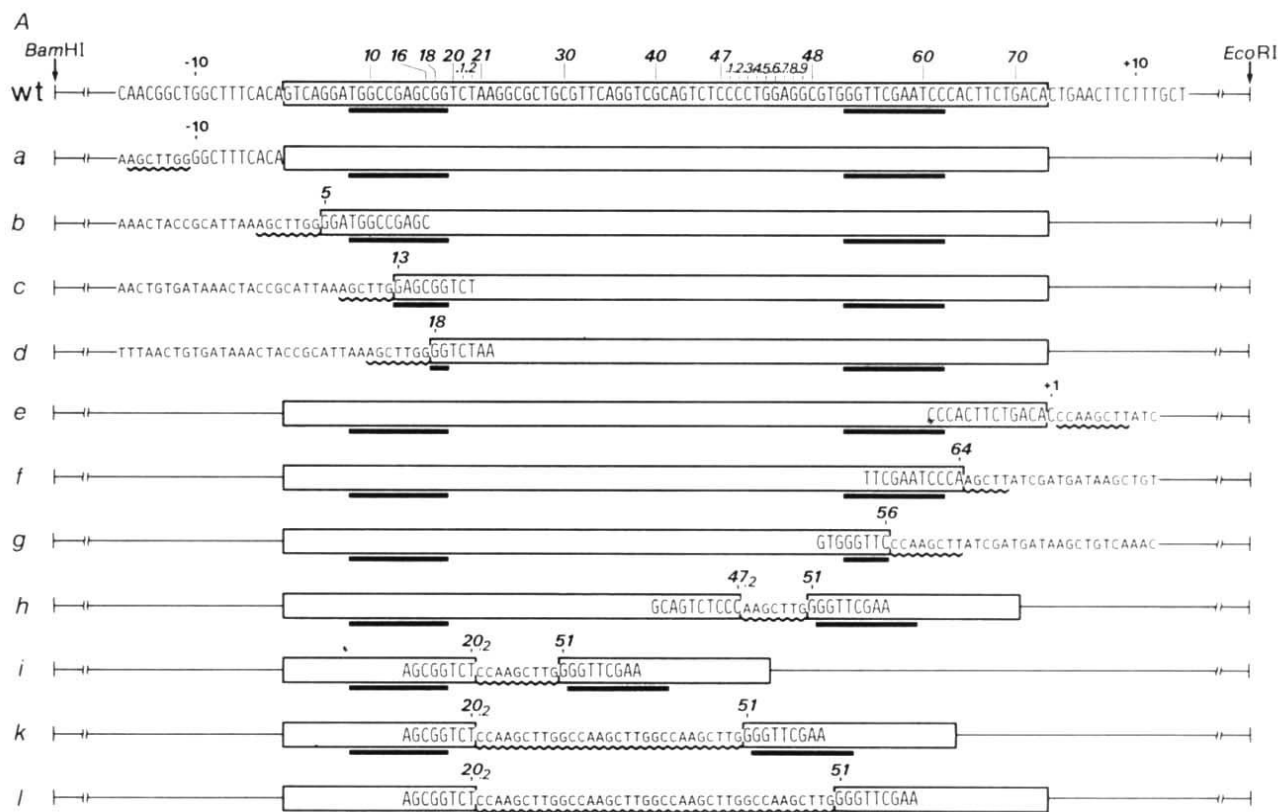
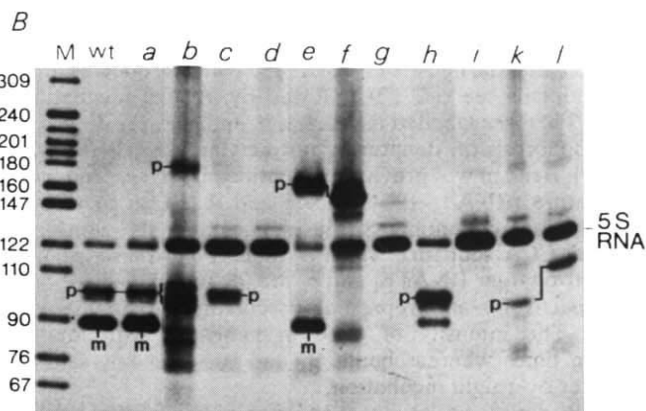


Fig. 3 Structure and transcription of the tDNA^{Leu} mutants. The starting material for the resection of the tRNA^{Leu} gene was the clone described in Fig. 2 legend. 15 µg of this plasmid were cleaved with *Eco*RI and another 15 µg with *Bam*HI. The linearized DNA was treated for 30 min at 30 °C with 0.2 U *Bal*31 double-strand exonuclease²³ in 20 mM Tris pH 8.1, 600 mM NaCl, 12 mM MgCl₂, 1 mM EDTA. In these conditions up to 200 nucleotides are resected from each end. 40 pmol of synthetic *Hind*III linker labelled with [γ -³²P]ATP were ligated to the *Bal*31-digested DNA, giving about a 15-fold molar excess of linker to DNA ends. Unreacted *Hind*III linkers were removed by centrifugation through a 5–20% sucrose gradient using a SW40 rotor. The plasmid DNA was precipitated and after resuspension in buffer digested with 80 U *Hind*III followed by *Bam*HI or *Eco*RI. The DNA fragments were separated on a preparative 8% polyacrylamide gel and eight gel slices were cut out containing DNA fragments ranging from 50 to 200 bp. After elution from the gel and chromatography on DEAE-Sephadex, the DNA fragments were cloned with pBR322 which had been previously cleaved with *Eco*RI and *Hind*III or *Bam*HI and *Hind*III and then treated with phosphatase²⁴. Recombinant plasmids were identified by restriction analysis and DNA sequencing. To produce insertion and deletion mutants, the individually cloned small subfragments were prepared from the recombinant plasmids by digestion with *Eco*RI and *Hind*III or *Bam*HI and *Hind*III followed by preparative polyacrylamide gel electrophoresis, elution from the gel and chromatography on DEAE-Sephadex. 1 pmol of pBR322 plasmid DNA which had been cleaved with *Eco*RI and *Bam*HI and treated with alkaline phosphatase was ligated with 1 pmol each of two corresponding DNA fragments. **A**, structure of the different tDNA^{Leu} mutants. The structural gene is boxed, *X. laevis* DNA sequences are shown in large letters, *Hind*III linkers and pBR322 sequences in small letters. *Hind*III linkers are underlined with wavy lines. Original sequences at the junctions which have been restored by the addition of *Hind*III linkers were considered as part of the gene unit. The bars below the sequence indicate the conserved sequence motifs. **B**, transcriptional analysis of the mutants carried out as described in Fig. 2 legend. Incubation time after injection was 3 h for wt and mutants *a*, *e* and *h*, and 15 h for mutants *b*, *c*, *d*, *f*, *g*, *i*, *k* and *l*. RNA from two oocytes was loaded on to the gel and the film was exposed overnight. Lane M, pBR322 plasmid DNA digested with *Hpa*II and end labelled with [γ -³²P]ATP. The length in nucleotides of the single-stranded DNA fragments is given to the left.



resection up to and including nucleotide 4 of the structural gene (clone *b*) deletes the normal initiation site but permits continued synthesis of several precursor molecules, albeit at a slightly reduced rate. S₁ mapping demonstrated that the majority of the transcripts from clone *b* initiate at the junction between plasmid and linker DNA and their heterogeneous length seems to stem from inefficient termination and incorrect processing (data not shown). The 5' border of essential promoter sequences can be placed around position 13, as clone *c*, which lacks 12 nucleotides of the structural gene, is still transcribed, although very poorly, whereas clone *d*, which lacks 16 nucleotides, is totally inactive.

Removal of the 3'-flanking sequences, including the putative termination signal TTCTTT (clone *e*), did not affect the rate of transcription but led to the synthesis of a 165-base precursor which, as deduced from its length, terminated within a run of Ts

located in the pBR322 plasmid DNA at positions 4,323–4,327 (ref. 14). A mutant which lacked 9 base pairs (bp) of the 3'-terminal sequences of the structural gene was transcribed at a reduced rate (clone *f*). However, a mutant lacking 17 bp was completely inactive (clone *g*). From the results of these two sets of experiments we conclude that the essential promoter sequences of the tRNA^{Leu} gene, detectable by oocyte injection experiments, are located somewhere between positions 13 and 64 of the structural gene.

The split promoter of the tRNA^{Leu} gene

To assess whether the gene-internal tDNA^{Leu} promoter is discontinuous, as is the case for the tDNA^{Met} promoter, we constructed mutants in which the central portion was replaced by a variable number of *Hind*III linkers. In one such tDNA^{Leu}

mutant the 14-bp region coding primarily for the extra arm of the tRNA^{Leu} (standardized positions 47.3 and 50) was replaced by a 10-bp synthetic *Hind*III linker DNA segment. This mutant was transcribed almost as efficiently as the wild-type gene (Fig. 3, clone *h*). Another mutant in which the 16 bp at positions 21–37 were replaced by 30 bp provided by three concatenated *Hind*III linkers, yielding an expanded gene, resulted in about 40% of the normal level (data not shown). In a third mutant, a segment 40 nucleotides long (positions 21–50) was replaced by one, three or four *Hind*III DNA linkers. The mutant containing one *Hind*III linker proved inactive in transcription (clone *i*). Substitution of the deleted sequences by three *Hind*III linkers reactivated the gene unit to a very small extent (clone *k*). Four *Hind*III linkers, which together exactly replace the length of the deleted sequences (clone *l*), activated transcription to a moderate level (discussed below).

Our previous experiments with the tRNA^{Met} gene⁵ showed that control sequences were located in the anterior and posterior portions of the structural gene. Considering the above substitution–deletion mutants of the tRNA^{Leu} gene as well as those created by 5' and 3' resection, a picture emerges which is more differentiated than that provided by the previous tDNA mutants. All four boundaries of the two gene-internal promoter

blocks have now been determined with some precision. By comparing the autoradiographic signals of the tRNA transcripts with that of the 5S RNA control, a rough estimate of the differences in tRNA production by the various injected clones can be made. Resection of the tRNA^{Leu} gene to position 5 is found to reduce transcription to a small degree (but curiously the product is now polydisperse and transcription termination is affected), whereas removal of sequences up to nucleotide 13 reduces transcription by a factor of about 20. Transcription is abolished altogether when the sequences up to and including nucleotide 16 are removed. This is in agreement with the tRNA^{Met} mutants, where resection up to nucleotide 8 had little effect but resection up to nucleotide 12 reduced transcription to below the levels of detection.

Deletion of all 3' sequences, including the termination signal, up to +1 of the sequences located 3' to the structural gene, nevertheless permits transcription at a level of 70% of control. When the terminal 9 bp of the structural gene are removed up to and including position 65, transcription continues, albeit at a fourfold reduced level, but deletion of the next eight nucleotides completely abolishes transcriptional activity. The results therefore suggest that transcription is reduced as resection of sequences approaches the two gene-internal conserved

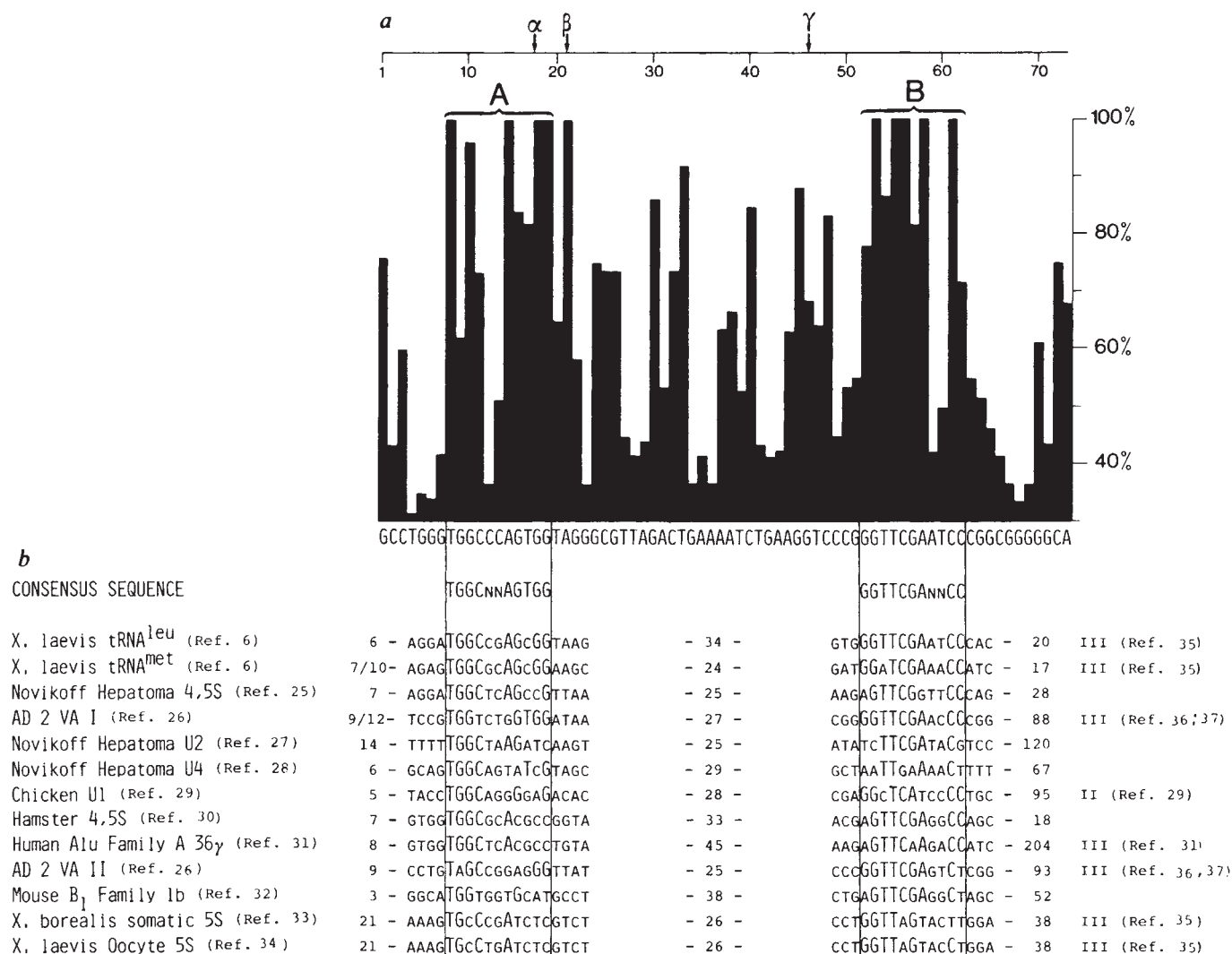


Fig. 4 Conserved sequence blocks A and B. **a**, The profile shows the frequency of occurrence of the most common nucleotide at every position in eukaryotic tRNAs as derived from a compilation of 80 eukaryotic tRNA sequences⁷. The sequence of the most frequent nucleotides is given below the profile. The arrows (α , β , γ) above the profile indicate discontinuities in the compiled tRNA sequences. α , Nucleotide 17 is omitted because it is only present in 40% of the sequenced tRNAs. β , Nucleotides 20 and 21 are separated by a variable number of nucleotides; thus, nucleotide 21 is not included in the A block. γ , Location of extra arm. The consensus sequence for the A and B block includes all nucleotides which occur with a frequency of over 60%. Sequences of selected small cellular RNAs were taken from the references given on the left. They were aligned for maximal homology to the A and B block region. Nucleotides matching with the consensus sequence are shown with large letters. The numbers given on the left and on the right of each sequence represent the numbers of nucleotides to the initiation and termination points, respectively. The numbers in the middle show the number of omitted nucleotides. The Roman numbers to the right of several sequences indicate which of the RNA polymerases transcribes the gene.

sequence blocks (underlined in Fig. 3; see below) but falls off precipitously when sections of these sequence blocks are removed. The internal sequences 21–37, as well as 47.3–50 can be replaced by *Hind*III linker and still support transcription of at least 40% of the wild-type level. This may be compared with the related tRNA^{Met} mutants, where substitutions of nucleotides 30–50 by *Hind*III linkers still permitted tRNA transcription, although at a reduced rate. These experiments show that gene sequences 21–50 can be replaced by foreign DNA, thus delimiting the gene-internal boundaries of most essential sequences. Note, however, that substitution of the entire sequence from 21 to 50 by *Hind*III linkers strongly inhibits transcription.

Thus, deletion and substitution of DNA segments around nucleotides 10–20 and 51–64 produces the most pronounced inhibition of transcription. Substitutions of small segments between these sequence blocks have smaller but nevertheless deleterious effects on transcription and these effects are apparently potentiated when several segments are removed together, as is the case for the 21–50 substitution. A picture therefore emerges for the tRNA^{Met} and the tRNA^{Leu} gene in which nucleotides particularly important for transcription promotion are seen to be clustered in two sequence blocks. Our unpublished findings that a minimal gene consisting entirely of DNA linkers and the above sequence blocks proved inactive in transcription indicate that these sequence blocks are necessary, but not sufficient, for initiation of tDNA transcription.

Conserved sequences in eukaryotic tRNAs

Figure 4a displays a profile showing the frequency of occurrence of the most common nucleotides at every position in eukaryotic tRNAs, as derived from a recent compilation of 80 tRNA sequences⁷. Two regions at positions 8–19 (A block) and 52–62 (B block) are highly conserved, with several nucleotides occurring at a frequency of 100%. The regions outside the A and B blocks exhibit a lower degree of nucleotide preference. Conservation of these sequences in both prokaryotic and eukaryotic tRNA sequences has previously been interpreted solely in terms of their importance to tRNA structure and hence protein synthesis; for instance, in the yeast tRNA^{Phe}, positions 8, 9, 14, 15, 18, 19 and 54–56 are essential in stabilizing the tertiary structure of the tRNA molecule¹⁵. Our present work on the tRNA^{Leu} gene has demonstrated that virtually all the nucleotides which are external to the conserved A and B blocks can be removed without eliminating transcription, while conversely, deletion of the conserved sequences abolishes transcription altogether. Hence, nucleotides in the A and B block have not only a function in tRNA structure but also in tRNA gene transcription.

If the conserved A and B blocks are the principal, functionally distinct promoter sequences, these blocks should be exchangeable between different tRNA genes without affecting their transcriptional activity. To test this idea we constructed two chimaeric genes which contained the 5' half of one and the 3' half of the other from the tRNA^{Met} and the tRNA^{Leu} structural genes and flanking sequences. Both chimaeric genes (Fig. 5, lanes 3, 4) were transcriptionally active in the frog oocyte. This focuses our attention again on the sequences held in common between these two tRNA genes and further suggests that the self complementarity of the acceptor stem sequences of tRNA genes is apparently not an important aspect of the tDNA promoter structure.

Another prediction of our model is that prokaryotic tRNA genes exhibiting strong A and B homologies should yield specific transcripts when tested in an eukaryotic transcriptional system. This was found to be the case for the tRNA^{Asp} and the tRNA^{Tyr} of *Escherichia coli* (W. Folk, unpublished data). Conversely, the *E. coli* tRNA^{Tyr} gene is not transcribed when injected into frog oocytes (cited in ref. 16), and this prokaryotic gene shows poor homology to the A block sequence. All these results strengthen our hypothesis that the two conserved homology blocks are important for the transcription of eukaryotic tRNA genes.

Diversity of promoters for polymerase III

If we consider all bases occurring at a frequency of at least 60% within sequence blocks A (positions 8–19) and B (positions 52–62) in over 80 different eukaryotic tRNAs, a 'consensus sequence' TGGCANNAGTGG (A block) and GGTTCGANNCC (B block) can be abstracted. Note that in some tRNA genes one or two additional nucleotides interrupt the A block at position 17. Therefore, the last two Gs of the A block either do not need to be co-linear with other nucleotides or are dispensable for transcription in some tRNA genes. Fowlkes and Shenk reported that two such sequence blocks also occurred in the adenovirus VAI and VAI gene and the 4.5S RNA gene of hamster cells. After mapping essential promoter sequences somewhere between positions 9 and 72 in the VAI gene⁴ they speculated that this sequence motif may be involved in transcription of RNA polymerase III genes. This hypothesis has now been shown to be correct for the tRNA^{Leu} gene of *X. laevis*.

The A and B sequence homologies can be extended to other genes transcribed by RNA polymerase III, for instance, to members of the human *Alu* and the mouse B1 families (Fig. 4b). Surprisingly, similar conserved blocks are also found in some of the genes coding for the U-RNA series, which code for capped RNAs and are therefore generally assumed to be transcribed by RNA polymerase II. The B block homology is particularly striking for many of these genes and it would therefore be worthwhile to investigate the potential of these genes to be transcribed by RNA polymerase III. Note that for most of the genes listed transcription initiation occurs 10–13 bp 5' to the A block, and in all cases (except for the 5S RNA genes) within 7–16 bp 5' to the A block boundary.

In Fig. 4b the genes have been arranged in order of diminishing strength of A block homology. This places the *X. laevis* oocyte 5S RNA gene clearly at the bottom. It is doubtful whether the A block homology of the 5S RNA is actually statistically significant. Dissection experiments² have shown the 5' portion of the gene to be largely dispensable in transcription promotion. However, more recent competition experiments also implicate the whole area from –11 to 28 (rather than a specific A block segment) in some transcriptional role¹⁷. The internal control region of the 5S RNA gene positions 50–83, although containing the B block homology, is considerably

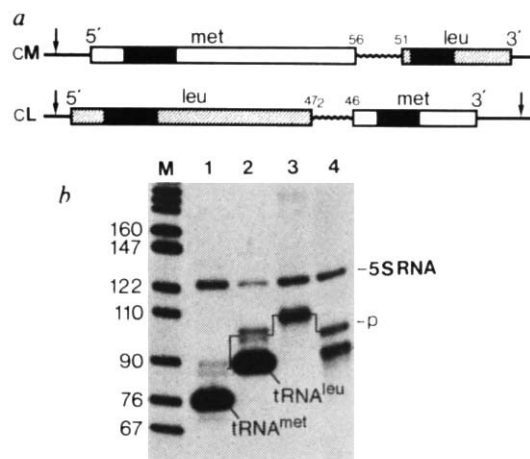


Fig. 5 Transcription of chimaeric tRNA genes. *a*, The chimaeric gene cM was constructed by combining, via *Hind*III linkers, the 3' half of mutant *h* (Fig. 3A) of the tRNA^{Leu} gene with a tDNA^{Met} fragment containing 120 bp of 3'-flanking sequences and the first 56 nucleotides of the structural gene⁵. cL is a combination of the 5' part of mutant *h* (Fig. 3A) of the tRNA^{Leu} gene with a tDNA^{Met} fragment which contains 28 bp of the 3' end of the structural gene and stretches 90 bp into the 3'-flanking sequences⁵. Wavy lines represent *Hind*III linker DNA, black boxes show the location of the conserved sequence blocks, arrows at the 5' end and 3' end indicate initiation and termination points, respectively. *b*, Lanes 1–4, transcriptional analysis of wt and mutant genes. Incubation time after injection was 15 h. RNA from two oocytes was loaded on to the gel and the film was exposed overnight. Lane 1, tRNA^{Met} wt; lane 2, tRNA^{Leu} wt; lane 3, chimaeric gene cM; lane 4, chimaeric gene cL.

larger than the posterior control region of tRNA genes. Therefore, both the primary structure of the 5S RNA and the tRNA genes and their response to sequence manipulation suggest that there are at least two subclasses of RNA polymerase III promoters (see also below) which differ from one another in their arrangement and the relative importance of specific sequence blocks. Other genes known to be transcribed by RNA polymerase III seem to fall between these two extreme cases. The above grouping of the genes is further supported by the results obtained from cofactor separation. Faithful transcription of the *Xenopus* tRNA^{Met} and the adenovirus VAI gene depends on similar protein fractions¹⁸, whereas the 5S transcription requires an additional cofactor which binds to the intragenic control region 50–83^{19,20}.

From previous experiments with the tRNA^{Met} gene unit⁵ it can be calculated that the minimal distance between the A and B blocks (see Fig. 4b) is about 29 bp. The tRNA^{Met} gene is one of the smallest genes transcribed by RNA polymerase III and in this gene the A and B block probably lie together in the closest proximity compatible with efficient promotion of transcription. However, the distance A–B in natural genes can be much larger, for instance, 41 bp in the *Xenopus* tRNA^{Leu} gene or 68 bp in the

yeast tRNA^{Leu} gene, which contains an intron²¹. It is therefore not surprising that the RNA polymerase III gene system can cope with tRNA^{Met} genes distended by 30 bp^{1,3} or with introns of the tRNA^{Leu} gene of yeast, into which additional sequences have been inserted²¹. The intermediate coding regions of tRNA genes located between the conserved A and B blocks include the sequences coding for the anticodon loop, the extra arm of different length (see Fig. 1) and the insertion sites of naturally occurring introns. It is therefore this region that predominantly differs between the various tRNA molecules. In keeping with this, the split tRNA promoter permits variability not only from single base changes, but also from extension or shortening of the sequences lying between the A and the B block. This flexibility contrasts with the 5S RNA gene operator system where only a single major type of RNA is produced and where transcription initiation occurs at a fixed distance from a single gene-internal control sequence.

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Human immunoglobulin D segments encoded in tandem multigenic families

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A family of germ-line immunoglobulin D-region genes has been cloned and mapped at regular intervals along a 33-kilobase length of human chromosomal DNA. Each member of the family varies slightly in sequence, but precisely conserves the recombinational signals and spacing that flank each gene. This region seems to have been formed by the tandem amplification of large and still well conserved segments of genomic DNA. Further, structural comparisons of germ-line and rearranged D segments suggest that D segments may recombine with each other.

THE genetic information necessary to form an immunoglobulin molecule is encoded in separate segments of DNA that must be brought together to form a coherent gene^{1–8}. This process occurs in developing B lymphocytes^{9–11} and is a central mechanism necessary for the creation of antibody diversity. Mouse immunoglobulin κ -chain genes, for example, are formed by joining one of several hundred germ-line V-region segments^{12,13} to one of four active J regions^{14,15}. These segments can be joined in different combinations and at slightly different cross-over points so that there are thousands of different possible end products^{14–16}.

Such recombination is thought to be mediated by special signals encoded 3' to each germ-line V-region sequence and 5' to

each functional J-region sequence^{6–8,14,15}. These signals are complementary to one another and consist of a small palindromic heptamer separated by an approximately 12- or 23-base spacer from an A- or T-rich nanomer. Thus, the consensus signal sequence, 5'-CACAGTG-12 base spacer-ACAAAACC-3', occurs 3' to each V_κ gene, while the consensus sequence, 5'-GGTTTTTGT-23 base spacer-CACTGTG-3', occurs 5' to each J_κ sequence. The sequences of the spacer regions are not conserved but their lengths apparently are. The conservation of these elements prompted the formulation of three rules for V–J recombination: the two signal sequences, each consisting of a nanomer and a heptamer part, must be complementary to each other^{14,15}; recombination can occur only between a signal of 12

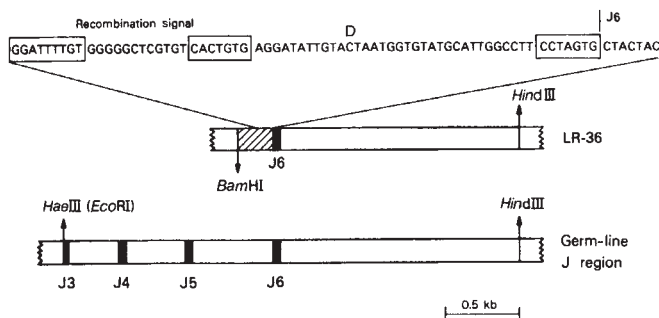


Fig. 1 Germ-line and aberrantly rearranged (LR-36) restriction fragments used for screening a Charon 28 phage library constructed from a partial *MboI* digest of human placental DNA. The novel DNA adjacent to J6 in LR-36 is depicted by cross-hatching; the nucleotide sequences comprising a D-like element are shown above. The LR-36 *BamHI*-*HindIII* fragment (1.9 kb) isolated from a pBR322 subclone was nick-translated to a specific activity of 200–500 c.p.m. per μ g as described previously³² and used to screen the phage library according to the Benton–Davis procedure³³. Fifteen hybridizing plaques were obtained and were rescreened with the embryonic *HaeIII*-*HindIII* fragment (~3 kb) in order to select those library clones that specifically hybridize to the novel germ-line D-like DNA shown by cross-hatching. (The embryonic *HaeIII* site was transformed into an *EcoRI* site by joining to the arm of the phage vector Charon 4A²⁰.)

base pairs (bp) spacing with one of 23 bp spacing^{7,8} and joining occurs near the ends of the heptamers, thus deleting the signal sequences in the recombinant^{14,15}.

Formation of heavy-chain V-region genes follows these same basic principles but is somewhat more complex; it involves the joining of three separately encoded elements, a V and J sequence as well as an additional element that appears between them^{7,8}. The additional segment, called D (for diversity), encodes a major portion of the third hypervariable region of heavy chains^{7,8,17}. Because ~23-base spacers were found in the recombination signals that flank germ-line mouse *V_H* and *J_H* sequences, it was predicted that germ-line D segments would be flanked by recombination signals separated by ~12-bp spacers. Two initial examples in the mouse bear out this prediction^{18,19}.

We set out to find and characterize such D-gene segments in man and to elucidate their organization and the extent of their representation in the human genome. The possibility of assembling V regions from three separate segments greatly increases the potential for generating diversity, but this potential immediately depends on the size of the germ-line repertoire of V, D and J region segments as well as the nature of the recombination event.

Our first requirement in any strategy designed to characterize germ-line D segments was to obtain a DNA probe containing germ-line D sequences so as to screen human genomic libraries. This posed a problem, because V–D–J recombinants generally contain only very short stretches of sequence derived from germ-line D segments and therefore are unsuitable as probes for screening. One way of circumventing this difficulty would be to isolate an intermediate in which the D segment had been joined to only one of its two eventual recombination partners, either the V or J segment. In this way, a longer segment of the germ-line sequence flanking the D segment would remain associated with the new V–D or D–J segment. We found such an intermediate, albeit somewhat aberrantly rearranged, in an IgM-bearing leukaemic lymphocyte in which both chromosomes had undergone rearrangement²⁰. One chromosomal copy, the active one, had been produced by V–D–J recombination (LR-35); the other (LR-36) had been generated by joining a D-like segment to the 3'-most *J_H* sequence, J6, to produce a D–J-type intermediate²⁰. The 5' portion of this clone became our probe for a D-region family. We show below that this D segment is apparently encoded in a large although closely related family. Members of this family are widely but evenly spaced over a 33-kilobase (kb) segment of

germ-line DNA, separated from one another by about 9-kb stretches of DNA. Surprisingly, these stretches are tightly conserved. The D segments vary slightly in sequence but retain their recombination signals. In addition, the structure of recombined D segments suggests that they might be shuffled, that is, recombine with each other, thereby further diversifying the third hypervariable region of heavy chains.

D-gene segment has aberrantly recombined with a J region

Lymphocytes from patients with chronic lymphocytic leukaemia seem to represent the clonal expansion of a single differentiated cell. Both chromosomes of one such IgM-bearing leukaemic lymphocytic clone (CLL-LR) have rearranged with respect to the *J_H* locus as discussed by Ravetch *et al.*²⁰. One recombinant (LR-35) is presumed to be the active gene, giving rise to an expressed IgM molecule. The other recombinant (LR-36), however, has been generated by joining a D-like sequence with the 3'-most *J_H* sequence, J6. This rearrangement is incomplete because V-gene sequences are lacking, and, furthermore, it is aberrant, because stop codons are located in phase with the J-coding frame. The new sequences brought in next to J6 include a recombination signal with a 12-bp spacer, as would be expected of such signals for the germ-line D segments^{7,8,9} (see Fig. 1). Consequently, this aberrant rearrangement seems to represent a germ-line D-gene-like sequence joined to J6, thus providing us with a larger probe for germ-line D-gene segments.

To obtain germ-line D segments, we screened a human placental library constructed in the vector Ch28 by partial *MboI*

Fig. 2 Southern blot analysis of human placental DNA (a) and of fibroblast (b) and lymphocyte (c) DNA from a leukaemic patient. Human DNAs were digested with the restriction enzyme *BamHI*, fractionated on a 0.8% agarose gel and then blotted on to nitrocellulose filters as described previously²¹. Filters were hybridized to the 1.8-kb germ-line *BamHI* fragment shown; this restriction fragment was derived from phage A3 shown in Fig. 3 and subcloned into pBR322. a, The probe hybridizes to itself (1.8-kb fragment) in addition to four more fragments 18, 7.4, 6.6 and 1.6 kb long. c, The germ-line D probe only hybridizes to those fragments located 5' to the D1 sequence which rearranged aberrantly with J6 (LR-36). Only the 18-kb and 6.6-kb fragments have been retained during DNA rearrangement in this B cell. (The rearranged fragment carrying the aberrant DJ6 recombinant (LR-36) is visible only after a long exposure as an about 12 kb band.) The fibroblast line (b) serves as a control and shows the same pattern seen in placental DNA (a).

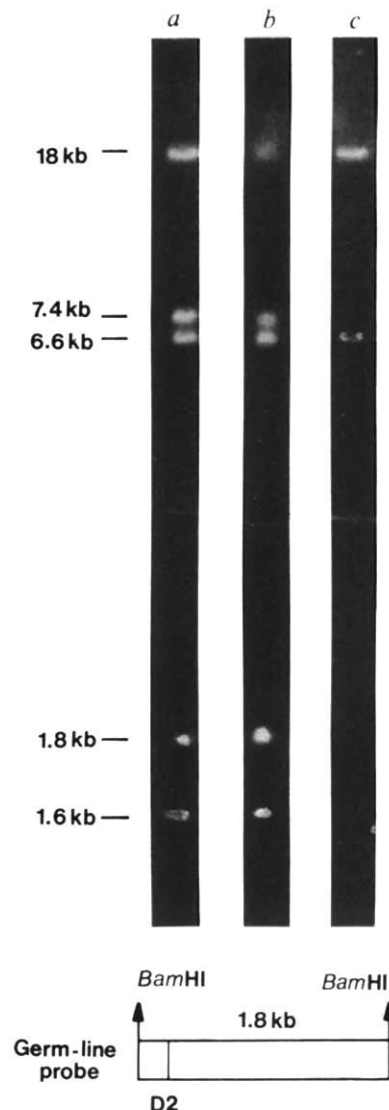
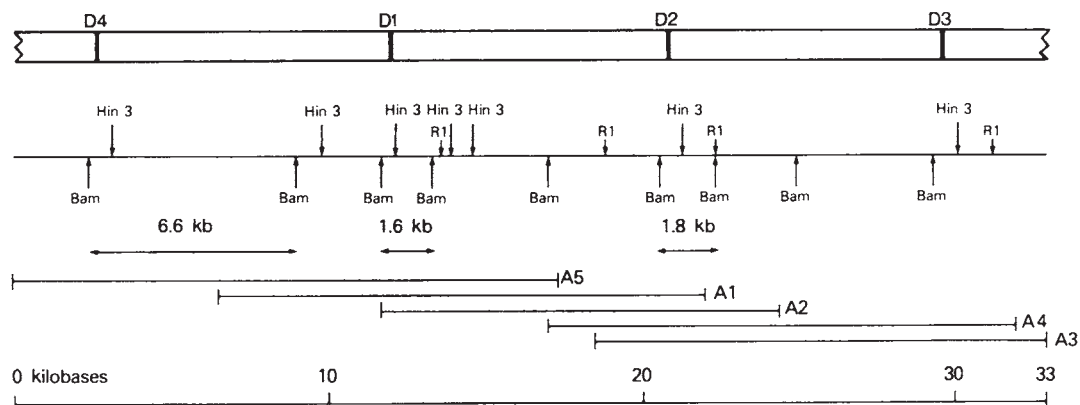


Fig. 3 Map of human D locus linking four separate D-gene segments on 33 kb of genomic DNA. Shown are a partial restriction map, the approximate extent of phage clones used to construct this map and the three genomic *Bam*HI fragments contained entirely on our clones (6.6, 1.8 and 1.6 kb long). D3 is located on a 7.4-kb genomic *Bam*HI fragment (see text) which is only partially contained on the phage A3. Phage clones A1–A5 overlap, whereas phage clone A6, containing only one D element (D5), has not been linked. The map illustrates the regular tandem arrangement of members of this family of D segments.



digestion. The fragment used as a probe is the 1.9-kb *Bam*HI–*Hind*III fragment shown in Fig. 1, a D–J probe isolated from the aberrant recombinant LR-36. Fifteen hybridizing phage clones were isolated and then rescreened with an embryonic J probe (*Eco*RI–*Hind*III fragment in Fig. 1). Only those phage clones hybridizing to the D–J fragment but not to the embryonic J probe contain germ-line D-gene segments. In this way we obtained seven phage clones containing D-gene segments.

D-gene segments exist as a family in germ-line DNA

To characterize the phage clones containing D-gene segments, the cloned DNAs were digested with *Bam*HI and hybridized with the D–J probe (*Bam*HI–*Hind*III in Fig. 1) according to the Southern blotting technique²¹. Such analyses revealed multiple *Bam*HI fragments with D segments. A characteristic 1.8-kb D-segment-bearing *Bam*HI fragment, found in four clones (A2, A3, A4 and A7), was subcloned into pBR322 and used as a germ-line D probe.

To confirm the appearance of multiple D-gene segments in the genome we digested total human placental DNA with *Bam*HI and probed it with the 1.8-kb germ-line D fragment. As seen in Fig. 2a, this probe detects not only itself, but also four additional *Bam*HI fragments of about 1.6, 6.6, 7.4 and 18 kb length. We conclude that D genes are indeed multiply encoded within the genome and are organized into gene families, reminiscent of V-gene families.

D genes are tandemly arranged on the genome

To learn more about the organization of D-gene segments we have mapped our phage clones with the restriction enzymes *Bam*HI, *Hind*III and *Eco*RI. Two phage isolates are indistinguishable from each other (A7 and A4) and five different phage inserts overlap each other. These overlapping phages link four different D genes to each other on a 33-kb long DNA segment, as shown in Fig. 3. One additional phage, A6, is as yet unlinked and contains one D segment.

The map of this D-gene family locus reveals a surprisingly regular tandem arrangement of D genes, separated from one another by about 9 kb. It is not known whether these large spacer regions are functionally significant. Three D-containing genomic fragments of 1.6, 1.8 and 6.6 kb, detected in a *Bam*HI digest of total placental DNA, are also present in our phage inserts. The remaining two genomic fragments of about 7.4 and 18 kb are likely to carry D3 and D5, located on the phages A3 and A6, respectively. In both cases, the clone inserts end within the genomic *Bam*HI fragments. Consequently we have cloned all five identifiable members of this D-gene family, four of which are regularly spaced ~9 kb apart.

We established the 5' to 3' order of these gene segments by analysing genomic DNA from chronic lymphocytic leukaemia

cells (CLL-LR). As discussed above, these differential leukaemic B cells have rearranged both chromosomes to give rise to an active (LR-35) and to an aberrant (LR-36) recombinant. Assuming that recombination is accompanied by a deletion of intervening DNA²² and that D genes reside between V and J genes, the chromosome carrying the active V–D–J gene should not contribute any germ-line D-gene fragments. The chromosome carrying the aberrant D–J6 recombinant, however, is expected to have retained those germ-line D-gene fragments located 5' to the aberrantly recombined D, while deleting those fragments located 3'.

Accordingly, we probed a *Bam*HI digest of DNA from CLL-LR with the 1.8-kb *Bam*HI fragment which contains germ-line D sequences; a DNA digest of a fibroblast line established from the same patient served as a germ-line control. As Fig. 2c shows, the D-bearing germ-line fragments of 1.6, 1.8 and 7.4 kb length have been deleted in the leukaemic B cells relative to the germ-line pattern (Fig. 2b). This orders the retained 6.6-kb long, D4-bearing fragment to the 5' side of the deleted 1.6-kb (D1), 1.8-kb (D2) and 7.4-kb fragments (see Fig. 3). According to the map order in Fig. 3, D3 must be deleted along with D1 and D2 and must therefore reside on the 7.4-kb germ-line fragment. (The only other genomic band, 18 kb long and as yet unlinked, is not deleted in this B cell.)

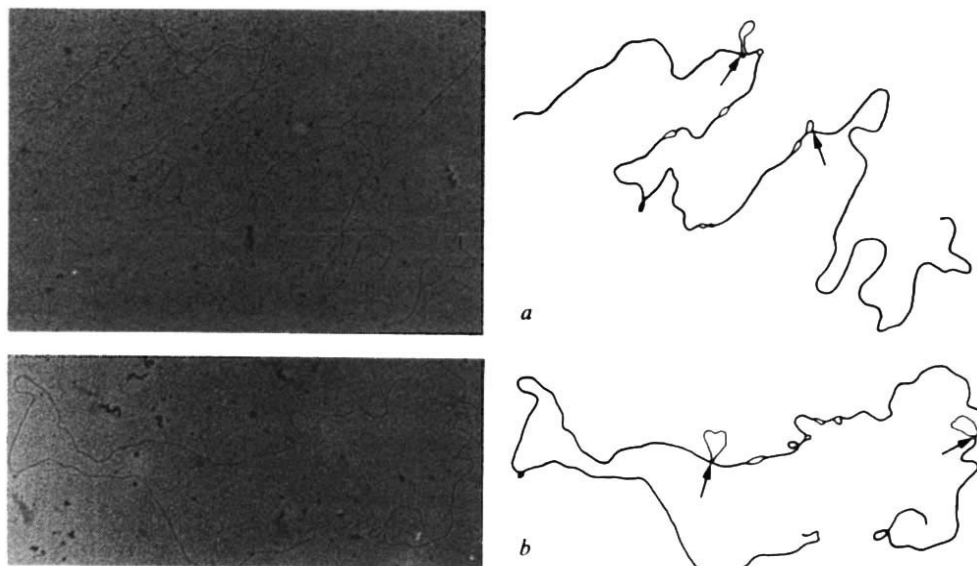
D regions are embedded in 9-kb repeat units of DNA

To identify possible homologies around D-gene segments, we heteroduplexed phages A5 and A3 with each other. Inserts within A5 and A3 do not overlap and so any heteroduplex formed between them is indicative of sequence homology, but not identity. Surprisingly, the two phages form a heteroduplex structure not only around the D segments, but almost throughout their entire lengths (see Fig. 4a). This extensive homology is interrupted only by several small nonhomologies in addition to deletion loops at the ends of inserts; such deletion loops are expected because different clones are unlikely to end in similar positions within the homology. Several of these small regions of nonhomology vary in size between molecules and occasionally are not visible, suggesting that even these regions have some homology, although not usually sufficient for heteroduplex formation.

Similarly, we can demonstrate homology between all the spacer regions surrounding these D segments. For example, the overlapping phage inserts of A3 and A2 (see Fig. 3) heteroduplex in a way which shows the spacer homology rather than identity of sequence in the overlap; such a heteroduplex is shown in Fig. 4b.

This locus of the human genome then appears to consist of 9-kb long repeat units, apparently arranged end to end, with each unit encoding one D-gene segment of this family. Such an organization is likely to be the result of very recent duplication

Fig. 4 Electron micrographs and schematic representations of heteroduplexes formed between phage clones containing D segments. Methods for heteroduplex formation have been described elsewhere³⁴. *a*, Phages A5 and A3 form an extensive heteroduplex, although their inserts do not overlap (see Fig. 3). Spacer DNAs between these D segments are homologous to each other. *b*, Phages A2 and A3 similarly form an extensive duplex. Pairing of the imperfect, though large spacer homology is preferred over pairing of the smaller region shared by these two overlapping phages (see Fig. 3). The spacer homology for both *a* and *b* is interrupted by a few small mismatched regions. The extent to which some of these small mismatch loops form varies slightly between heteroduplex molecules, although the overall pattern of these mismatch regions is preserved and is found reproducibly in more than 10 molecules examined. Phage insert end are indicated by arrows. Deletion loops at the insert ends merely show that the two phage clones do not end at identical positions within the homology.



events. Nonetheless, mechanisms that operate to maintain sequence homologies within the spacer cannot be ruled out.

Germ-line D-gene segments are surrounded by recombination signals with 12-bp spacers

To establish formally the existence of D segments on our clones and to allow further characterization, we sequenced the relevant regions using the Maxam-Gilbert procedures. Figure 5 shows four such embryonic D sequences of this family. All are surrounded by recombination signals with a 12-bp spacing between the heptamer and nanomer sequences. This allows for recombination with signals of about 23 bp spacing, as are found on the 3' side of V_H genes^{7,8,23-25} and the 5' side of J_H segments^{7,8,20,26}, and as would be required by the 12/23-bp spacing rule.

The sequences within and surrounding these D segments are closely homologous to each other, differing mostly by point mutations, except for a deletion of a triplet in D3. Most of the nucleotide differences lie within the potential coding sequences (that is, between the recombination signals) and in particular near the middle of these segments. The recombination signals and spacers, on the other hand, particularly those on the 5' side, are very strongly conserved. Surprisingly, the 5' and 3' spacer sequences are quite complementary to each other and, together with the complementary recombination signal, an extensive stem structure could be drawn, looping out the D-coding segment.

D sequences of different families are heterogeneous in length. The presumptive coding blocks of this family of D segments are 31 bp long (D3 has 28 bp). DHQ52, a D segment interspersed

among J regions in man and 5' to J segments in mouse, is 11 and 10 bp long, respectively^{18,20}; DSP2.2 in mouse encompasses 17 bp¹⁹. Observed size variations in the third hypervariable region of heavy chains²⁷ may reflect length heterogeneity of D segments.

The diversification of variable regions contributed to by D segments increases even further when we consider the inherent flexibility associated with recombination events^{14,15}. Flexibility in the exact point of joining has been demonstrated for V-J recombination in light chains; for example, V and J segments are sometimes joined out of phase with each other²⁸.

In contrast, D segments have no known coding frame. Therefore, if we assume a flexible recombination mechanism, all three reading frames of D sequences are potentially usable. This would further diversify immunoglobulins of different B cells. In this context, all three reading frames of D1 are open, that is, they do not contain any stop codons, while D2, D3 and D4 each have two open reading frames.

Is aberrant D-J the result of more than one recombination event?

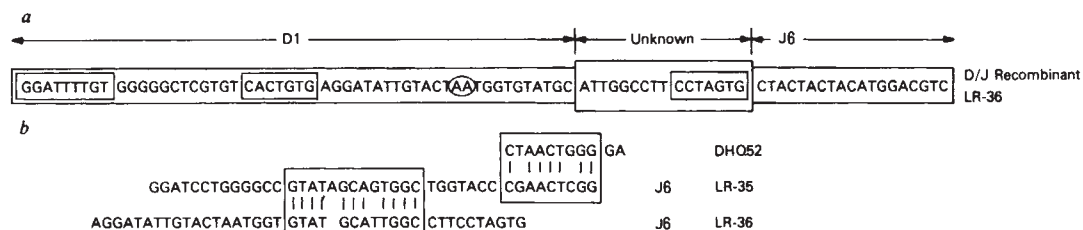
As discussed above, only D1 can be the parent of the aberrant D-J6 recombinant. Germ-line DNA between D1 and J6 has been deleted during recombination (Fig. 2c) and sequence analysis confirms this assignment (see Fig. 6a). Sequences within 200 bp 5' of D1 are identical to those 5' to the D-J6 recombinant, as determined by restriction mapping and partial sequencing. All other members of this family (including D5 on phage A6) exhibit similar, but clearly different sequences.

Fig. 5 Nucleotide sequences of four members of this human D family (D1, D2, D3, D4) in addition to DHQ52, published elsewhere²⁰. The D4, D2 and D3 sequences are compared with D1 and differences are shown by gaps or encircled bases. The

D Segments									
← 12bp →							← 13bp →		
GGATTTGT	GGGGGCTCGTGT	CACTGTG	AGGATATTGTACTGGTGGTATGCTATACC	CACAGTG	ACACAGCCCCATT	CCCAAAGCC	D1		
GGATTTGT	GGGGGCTCGTGT	CACTGTG	AGGATATTGTAGTGGTGGTGGTCTATCTCC	CACAGTG	ACACAGCCCCATT	CCCAAAGCC	D2		
GGATTTGT	GGGGGCTCGTGT	CACTGTG	AGCATATTGTGGTGGTGGTGGTCTATCTCC	CACAGTG	ACACAGCCCCATT	CCCAAAGCC	D3		
GGATTTGT	GGGGGCTCGTGT	CACTGTG	AGGATATTGTAGTGGTGGTGGTGGTCTATCTCC	CACAGTG	ACACAGCCCCATT	CCCAAAGCC	D4		
GGTTTTGG	CTGAGCTGAGAAC	CACTGTG	CTAACTGGGGA	CACAGTG	ATTGGCAGCTCT	ACAAAAACC	DHQ52		

the D segments are boxed—all contain a 12- or 13-bp spacer between the heptamer and nanomer parts. No other recombination signals are found in the immediate flanking sequences (not shown here). All four D segments were sequenced according to the Maxam and Gilbert procedures³⁵. D1 was sequenced from a 400-bp long *Bam*HI-*Hind*III fragment, whereas D4, D2 and D3 were sequenced from 800-bp long *Bam*HI-*Hind*III fragments (see Fig. 3). (These fragments were obtained by restriction of subcloned *Bam*HI fragments (see Fig. 3).) D segments were located by random sequencing within these fragments. Sequences were determined in both directions. The four D segments are much more closely homologous on their 5' sides than on their 3' sides, although they all show extensive homology except near the middle of the coding segments. D3 has deleted a triplet whereas all other differences are point mutations.

Fig. 6 *a*, Parents of the aberrant D-J recombinant LR-36. The nucleotide sequence of LR-36 is derived entirely from the D1 parent and the J6 parent except for 16 bp immediately adjacent to J6 (designated 'unknown' here) and 2-bp mutations 10 bp 5' to the unknown DNA (circled here). This novel DNA contains a heptamer-like recombination signal and may represent the 3' end of an unidentified D segment which recombined with D1. Recombination signals are boxed. *b*, The active recombinant LR-35 sequence shows segmental homologies with the LR-36 sequence and the DHQ52 germ-line segment. Homology regions are boxed. LR-35 seems to derive parts of its sequence from D segments also used by the aberrant recombinant LR-36, but in addition shows homology to DHQ52. This suggests that different D segments may have recombined with each other to form a mosaic LR-35 sequence. (Interestingly, the region shared by LR-35 and LR-36 spans the presumed recombination point in LR-36 (see part *a* of this figure).)



Although most of the coding segment of D1 is preserved during recombination, the six 3'-most base pairs of D1, are not found in the recombinant. Instead, 16 bp of unknown origin appear directly adjoining J6 (see Fig. 6*a*). What gave rise to these sequences? As this sequence arrangement is not present in germ-line DNA, we must invoke an additional rearrangement: D segments may be able to recombine with each other. Within the 16 bp of unknown origin we find a heptamer—CCTAGTG—which resembles the heptamer consensus sequence for recombination signals. This may be a remnant of the 3'-recombination signal attached to an unknown D sequence which recombined with D1; joining to J6 did not completely eliminate the signal.

The parent sequence D1 and the recombinant LR-36 also differ in two adjacent nucleotides within the otherwise preserved D sequence (10 bp 5' to the presumed D-D joining site, see Fig. 6*a*). These point mutations might have arisen somatically, either connected with or independent of the recombination event. Also possible is a polymorphism in the DNA sequence of this patient. Although less likely, we cannot rule out the possibility that D1 recombined with another D 5' to the two non-matching nucleotides. This would require part of the unknown D segment to be identical with D1.

The presumed active recombinant LR-35 provides further, albeit indirect, support for the D-D recombination proposal. Part of the LR-35 gene sequence is quite homologous to a sequence in the D segment of the aberrant recombinant LR-36; beyond this homology region, the two sequences diverge. However, the LR-35 D segment also has some additional homology with another germ-line gene segment, DHQ52. This homology is 3' to the homology it has with LR-36 (see Fig. 6*b*), which suggests, but does not prove, that at least three different D segments have contributed to the LR-35 D; that is, that multiple recombinations between D segments can generate active D-region segments.

At present we do not know how D segments recombine with each other because no appropriately situated recombination signals exist to accomplish such joining. In light of existing recombination events, we would expect recombination signals con-

forming to the 12/23-bp spacing rule next to the eventual joining sites of the two D segments. We see no such signal next to the presumed site of rearrangement in the germ-line D1 sequence. On the other hand, it has been suggested that such a signal exists in some mouse segments²⁹. It is possible, however, that the extensive homology that surrounds the D-region sequences has a role in their interaction, either somatically or within the germ line. Such a mechanism, involving homologous recombination or gene conversion, would allow segments to amplify their germ-line diversity in much the same way as has been proposed for members of V-region families¹². Clearly, the resolution of this question will depend on the further analysis of germ-line and rearranged D-region genes.

D-gene segments can generate enormous diversity

The variable region of the heavy chain derives its diversity from four principal sources: multiply encoded V-, D- and J-gene segments; a combinatorial joining mechanism; flexibility in the exact site of recombination that can generate different amino acids at junctions between joined segments (in the case of D segments, this flexibility allows reading in different frames); and a potential for solitary somatic mutations, an additional process now being documented^{24,25,30,31}.

The most diverse portion of the heavy chain is the third hypervariable region. This is understandable in terms of what we know of D-region genes. Not only are D segments multiply encoded, but these short sequences of variable length might also recombine with each other. Flexibility at the site of joining further diversifies junctions between V, J and other D regions and could create different reading frames within D segments. This system is therefore able to generate an enormous amount of coding information, particularly within the third hypervariable region, providing a major source of diversity.

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LETTERS TO NATURE

HR 465 returning to rare-earth-maximum phase

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HR465 is unusual among Ap stars. Its spectroscopic variability has been discussed by Wolff and Preston¹ who show that at the rare-earth-minimum phase, the sharp-lined spectrum is similar to that of many other Ap stars of the 'magnetic sequence'. The rare-earth-maximum phase, studied Bidelman and Pyper (personal communication), was found to have an extremely high density of lanthanide rare-earth lines in addition to Hg II 3,984 Å. Their observations of this phase were made in the early 1960s and because of the very long period of the star, around 22–24 yr, they have not been repeated. Until recently, the critical phase of HR465 has been unavailable. However, we report three 2.4 Å mm^{-1} spectrograms of HR465 taken at the Dominion Astrophysical Observatory (DAO) in August 1981 which show that the spectrum now very closely resembles that of the early 1960s—the rare-earth-maximum phase has returned.

Aller and Cowley's² announcement of the possible presence of the unstable element promethium, whose longest-lived isotope has a half life of only 18 yr, in the spectrum of HR465 caused much interest. Setting aside this controversy^{3,4}, the rare-earth-maximum spectrum of HR465 is of great interest. The elemental abundance patterns^{5,6} suggest the presence of *r*-process peaks. Ohnishi⁷, and Steinberg and Wilkins⁸ have discussed possible interpretations of these abundances in terms of fission. There are strong arguments⁹ for the presence of the actinide rare earths uranium and thorium. An unusual distribution of wavelength residuals suggest that the uranium may contain a high percentage of isotopes lighter than 238. A possible uranium-to-thorium ratio greater than unity could be interpreted in terms of a recent *r*-process event, and the excess uranium would then be due to the presence of isotopes lighter than 238 (ref. 10).

The star has been monitored at the DAO since 1974. In 1979 and 1980, substantial increases in the line density were noted, as well as a progressive strengthening of lines of the heavier lanthanides, notably dysprosium. Particular attention was paid to the feature near 3,859.6 Å, which closely coincides with the wavelength of the strongest U II line¹¹. This feature, which is dominated by Cr I in most magnetic Ap stars, weakened throughout the late 1970s along with the Cr I and II spectra. There is a pronounced weakening of Cr I throughout this period.

In the August 1981 spectra, 3,859.6 Å has strengthened considerably; it may now be considered a moderately strong line (~80 mÅ). Figure 1 shows tracings of the region of U II 3,859.6 Å made from spectrograms taken at the previous rare-earth maximum (A), an intermediate epoch (B), and in August 1981. The top two tracings have been described and detailed line identifications given elsewhere¹². Figure 1C shows DAO plate no. 13891, taken in August 1981. Filled squares below the absorption lines mark features which have been wholly or partially identified as due to lanthanides. While most of the features in the rare-earth-maximum spectrum are marked with squares, only three features are so marked in Fig. 1B, where many of the absorptions are due to chromium.

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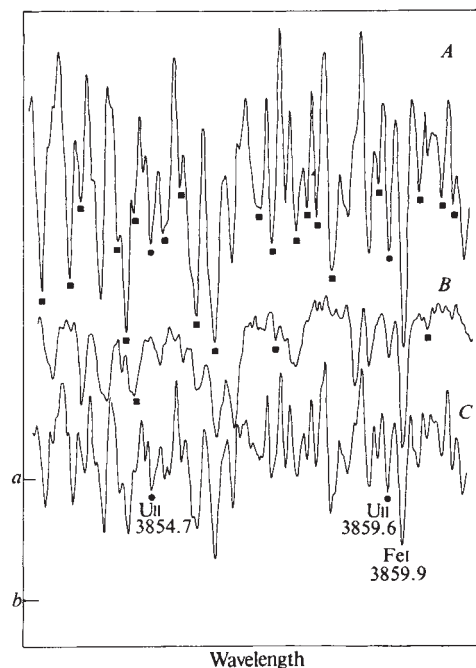


Fig. 1 Direct intensity tracings of HR465 taken on September 1961 (A), November 1974 (B) and August 1981 (C), in the region of the strong U II lines at 3,854.7 and 3,859.6 Å (●). The strong Fe I line is at 3,859.91 Å. Lanthanide lines are indicated by solid squares (■). A one-to-one correspondence of features in the A and C is apparent. Zero intensities for A and B are marked a and b respectively. The base is zero for C.

Wavelengths for one of the current DAO plates have been measured from PDS scans using a program developed by Rice. Positions for 4,143 lines in the region 3,707–4,655 Å were analysed by the method of wavelength coincidence statistics (WCS)¹³. Essentially the same heavy ion species that were present at the last rare-earth maximum can again be identified. In particular, dysprosium (Dy III is especially strong), Ho and Er are identified at very high confidence levels. The 1981 plate shows good statistical evidence for the presence of osmium, and marginal evidence (98% confidence) for platinum. The presence of both of these transition metals has been found at less exotic phases.

WCS of the 1981 plates show marginally significant results for U II (97.5% confidence), Th II (97.5% confidence) and Th III (99.5% confidence).

The present observational material (2.4 Å mm^{-1}) is not good enough to allow us to extract definitive information on the critical isotopic abundance ratios. Very high resolution and low noise are needed. Many features must be studied because of the severe problem of blends.

For the next few years, HR465 will be in a critical phase which deserves intensive investigation.

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Supernovae and nitrate in the Greenland Ice Sheet

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Nitrate concentration in the absolutely dated Greenland ice core from Crête has been measured for six time intervals, five surrounding the time of appearance of the well established historical supernovae during the past 1,000 yr and one during the Maunder minimum of solar activity, to look for a possible correlation between supernovae and nitrate concentration. The findings of Rood *et al.*¹ of nitrate spikes corresponding to the appearance of the historical supernovae and a pronounced minimum in nitrate contents during the Maunder minimum are not confirmed. A very regular annual variation of nitrate concentration is observed superimposed on a constant background. We show here that both these signals seem unaffected by the known variations in the solar activity for the periods analysed. The nitrate contents are unaffected by peaks in acidity caused by volcanic eruptions. The annual variation of nitrate concentration suggests it could be used for dating ice cores.

Nitrate deposited in the Antarctic Ice Sheet has attracted attention because of its possible production by the absorption of ionizing radiation from extraterrestrial sources in the stratosphere^{1–3}. The deposition rate of nitrate in snow accumulated in areas remote from soil and sea should then reflect the influx of radiation from space, modulated by atmospheric processes such as exchange between the troposphere and stratosphere and the general tropospheric circulation. If this were true, the concentration of nitrate in polar glacial ice may contain information on solar activity variations, galactic cosmic-ray influx and changes in the atmospheric circulation connected with climatic changes.

Rood *et al.*¹ have discussed whether the flash of X rays from a supernova explosion in our Galaxy might produce sufficient NO in the stratosphere to produce a spike of nitrate in ice cores a year or two after the event. In an Antarctic ice core they report four spikes with a factor of 2–3 higher than the background level. Three of the spikes seem to coincide reasonably well with the supernovae observed in 1181, 1572 and 1604. We know of no absolute dating for the South Pole station core in question and the expected accuracy of the dating is not reported by Rood *et al.*¹. Three proposed datings differ ~40 yr for the AD 1181 event. The fourth nitrate event, at about 1300, is not correlated with any known historical supernova. Rood *et al.* report further indications of variation of the background level with the solar cycle and claim a pronounced low level of nitrate coinciding with the Maunder minimum of solar activity.

The perspective of these observations and the availability of the absolutely dated Crête ice core, induced us to measure nitrate in the Greenland ice. The fact that the historical supernovae predominantly lighted the Northern Hemisphere suggests that detection is more likely in Greenland than in Antarctica.

Supernova explosions are rarely observed in our Galaxy: Table 1 lists the data for the five historical supernovae observed

in the past 1,000 yr. Data are partly historical and partly from recent optical and radio observations of the remnants⁴.

A supernova, Cas A, is believed to have occurred around 1750, but as the time of appearance is based only on extrapolation of present-day observations of the remnant it is not considered here. The next candidate before 1006 is AD 393 (ref. 4) which is out of range of the ice core material available for this study. However, the material does include the Crab supernova of 1054 and notably the year 1006 event, which is the one closest to the Solar System and the brightest new star ever observed.

The 404-m polar ice core used in this study was recovered using a thermal drill in a dry hole in Central Greenland 71°N, 37°W in 1974 by the US Cold Region Research and Engineering Laboratory under the US–Swiss–Danish program Greenland Ice Sheet Program (GISP). The core handling and storage procedures have been described by Langway⁵, and the sample preparation by Hammer⁶. For the time intervals in question the ice core is dated to an accuracy of ± 1 yr back to AD 1104 and ± 3 yr back to AD 553, which is the age at the bottom of the core. The dating is done by counting seasonal variations of the stable oxygen isotope ratio, $\delta^{18}\text{O}$, and acidity downwards from the surface⁷. The acidity curve provides the time scale with fix points back to AD 1104 using historical accounts of volcanic activity in the Northern Hemisphere. A volcanic eruption causes an increase of the acidity of the polar ice, the size of the signal found in the ice depends on the magnitude and geographical latitude of the eruption. All sample collections are based on continuous sequences of 12 ice samples per calculated annual layer for $\delta^{18}\text{O}$ and acidity, and in general of four samples per calculated year for nitrate. The theoretical thickness of an annual layer at a given depth is calculated from a flow model for the ice sheet⁷. The mean annual accumulation at Crête is 29 cm of ice and the thickness of a calculated year AD 1000 is ~25 cm. Extreme care was taken to ensure no contamination of the samples for nitrate analysis. Samples of about 10 ml were cut in a laminar flow bench and a microtome knife used to clean the ice surfaces. Several centimetres of ice were cut away from the surface of the core. The samples were taken from crack-free regions of the core. The clean samples were put into sealed plastic beakers and kept at -20°C . Melting was done just before the chemical analysis for nitrate.

The nitrate concentration (given as $\mu\text{equiv. NO}_3^-$ per kg ice) was measured as nitrite after reduction in a cadmium column. The nitrite was converted into a form of an azo dye by adding sulphanilamide and *n*-(1-naphthyl)-ethylene diamine dihydrochloride⁸. The optical absorption of the dye solution was measured. The reproducibility of the method, $\pm 0.02 \mu\text{equiv. kg}^{-1}$ ($\pm 2\%$), was achieved during the single runs of analysis. For comparison of the absolute levels between the six series of measurements an accuracy of 5% was obtained: the source of error was the uncertainty in the dilution factor for the standard solutions used for the calibration. The measured quantity is the sum of nitrate and nitrite. The concentration of nitrite was measured on a few of the samples by bypassing the cadmium reduction column. The contents were always consistent with zero within the quoted measuring accuracy. The measurements are therefore assumed to give the nitrate concentrations.

Figure 1 shows the results of the nitrate analysis for the six short series of the selected time intervals covering the years before and after the appearance of the historical supernovae listed in Table 1. Also shown are curves giving the acidity from conductivity measurements⁹ together with $\delta^{18}\text{O}$ data. The $\delta^{18}\text{O}$

Table 1 Parameters of five historical supernovae

Date	Name	Position (1950) RA Dec	m_v	Type	Distance (kpc)	Visibility
1006 3 April	—	15 10 –40	–9.5	I	1.3	2 years
1054 4 July	Crab	05 40 +20	–4	—	2.2	22 months
1181 6 August	—	01 30 +65	—	—	8	185 days
1572 8 November	Tycho	00 20 +65	–4	I	7	16 months
1604 8 October	Kepler	17 30 –20	–3	I	10	12 months

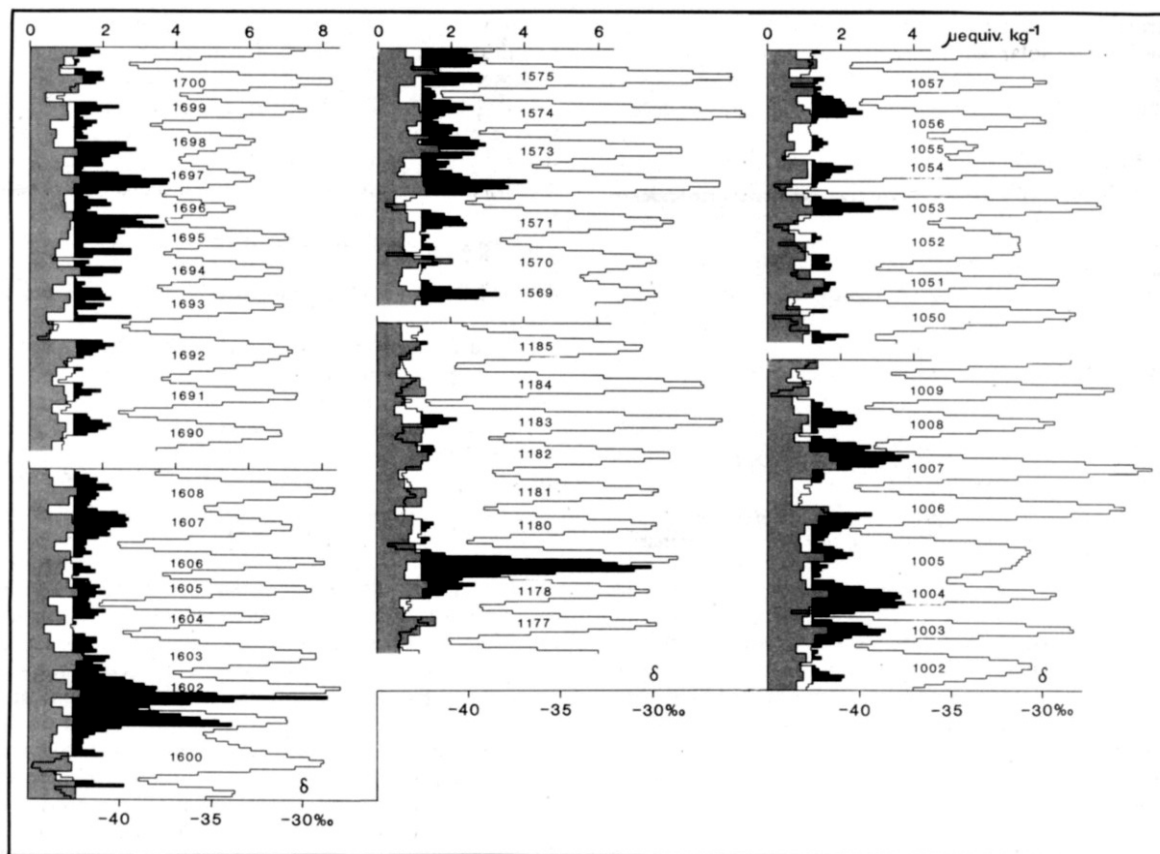


Fig. 1 Three curves for each of the six time intervals. Nitrate concentration (stippled area: left-hand scale $\mu\text{equiv. kg}^{-1}$). Acidity fallout (black area: left-hand scale $\mu\text{equiv. H}^+$ per kg ice). Acidity above the background $1.2 \mu\text{equiv. kg}^{-1}$ are due to fallout of volcanic acids, mainly H_2SO_4 and HCl , from eruptions in the Northern Hemisphere. $\delta^{18}\text{O}$ to the right with scale at the bottom (per 10^3).

curve is first order corrected for diffusion¹⁰. The beginning of a calendar year is arbitrarily defined as the point where the corrected $\delta^{18}\text{O}$ begins to rise. The nitrate curve is represented by a mean of four samples per calendar year. By distributing and assigning the measured amount of nitrate to the calendar years defined above, one arrives at the mean nitrate concentration in calendar years. Figure 2 shows the annual deposition determined in this way. At the end of each curve the mean value and the standard deviation of the annual values are indicated. The bars reflect the variability of the phenomenon, the experimental errors in the chemical analysis being an order of magnitude smaller. The uncertainty in placing the divisions between calendar years also contributes to the standard deviation. No significant enhancement beyond the normal variations in nitrate

deposition is observed in the years of the five supernovae or the years following these events. From the accuracy of the absolute dating we conclude that the analysed portions of the ice core cover the times of appearance of the five supernovae. The curve for the interval AD 1690–1700, which is selected to represent the Maunder minimum, shows that nothing unusual is taking place, the mean deposition of nitrate is at the same level.

The correlation between nitrate and $\delta^{18}\text{O}$ data is good. Like $\delta^{18}\text{O}$ the nitrate shows a regular annual variation that is clearly visible, although there are only four samples per calculated year. Both curves indicate concordantly the fluctuations in the annual amount of precipitation. The annual variation in nitrate deposition seems to be the same for the six time intervals observed, sampling a period of 700 yr. Both the variation and the mean

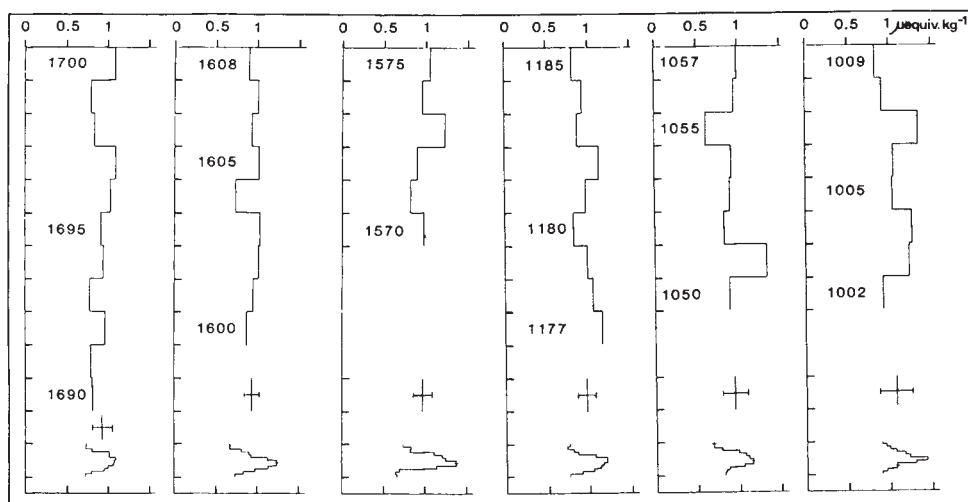


Fig. 2 Calendar annual means of nitrate concentration in $\mu\text{equiv. kg}^{-1}$. The bars indicate the formal standard deviation and mean value of the calendar values, that is they do not reflect the experimental precision of the chemical analysis. The inset at the bottom shows the mean annual variation calculated by signal averaging from the original data of Fig. 1.

level in nitrate deposition seem to be independent of the recognized variations in solar activity during the Maunder minimum¹¹. Figure 2 also shows the mean annual variation curves in nitrate deposition which were obtained by a signal averaging procedure¹². From the measurement in Fig. 1, 12-monthly values are read evenly distributed in each calendar year. Average values are then taken for each month. The curves indicate an increase in nitrate deposition in the spring and a decrease in the autumn. The trough to peak variation is $\sim 0.5 \mu\text{equiv. kg}^{-1}$ and the background is $\sim 0.75 \mu\text{equiv. kg}^{-1}$.

There seems to be no variation of the amplitude of the annual oscillation with an 11-yr sunspot cycle. The solar activity is, however, not well recorded in pre-telescopic time¹¹ and conclusions must wait for a comparison with nitrate data from a longer and more recent period.

The six time intervals contain two events in acidity ascribed to volcanic eruptions⁹. One is in AD 1178–1179 and correlated with an eruption of Katla, Iceland. The other is in AD 1601–1602 and not identified⁹. Note that the nitrate curve goes undisturbed through the volcanic peaks. The acidity peaks are mainly composed of sulphuric and hydrochloric acids⁹.

The regular annual oscillation in nitrate contents makes it in principle possible to date ice cores by observing the nitrate signal, especially in polar ice from regions where no summer melting occurs (such as at Crête) and thus no percolating melt water penetrates the snow and disturb the stratified layers. We suggest that the higher deposition rate of nitrate in the summer is due to a stratospheric fallout following the opening of an tropospheric–stratospheric exchange every spring. The modern nitrate deposition in rain water in areas of Scandinavia, remote from industrialized regions, is at the level of $2 \mu\text{equiv. kg}^{-1}$, and the natural pre-industrial deposition rate can be set to the level of $1 \mu\text{equiv. kg}^{-1}$ judged from the lowest observations in northern Scandinavia¹³. The winter level of deposition in the Greenland ice is of the same magnitude and is therefore consistent with a residual tropospheric contribution in the clean air conditions above the Greenland ice.

It has been suggested that supernovae occurring close to the Solar System will enhance production of NO in the stratosphere and consequently produce observable nitrate peaks in ice cores^{14,15}. As shown here the supernova (SN1006) occurring as close as 1,300 pc, caused no such effects in the Greenland ice. Candidates closer than that are the supernovae corresponding to the young short period Vela pulsar, the Lupus Loop, the Cygnus Loop and the North Polar Spur. The ages of these supernova remnants are estimated to be in the range 10,000–100,000 yr¹⁶. This matches the time interval for which glacial material is available from the Antarctic and Greenland Ice Sheets. A local bubble of hot interstellar material observed in soft X-rays is considered to have originated from a supernova having exploded very near (<18 pc) the Solar System (R. Rothenflug, M. Arnaud & A. Soutoul, personal communication and ref. 17). If nitrate events in ice cores could be found and correlated with close supernovae an accurate dating of these is possible using the glacial record, and information on emission of γ rays and cosmic rays and their propagation to the Solar System could be obtained.

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Deuterium/hydrogen ratios in unequilibrated ordinary chondrites

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The discovery¹ of oxygen of anomalous isotopic composition in the Allende meteorite during the early 1970s led to the detailed investigation of primitive materials for unusual isotopic signatures. One element studied was hydrogen; major enrichments in deuterium relative to terrestrial and proposed nebula compositions have been discerned by close examination of specific phases within various meteorite samples. Here we report high and variable deuterium enrichments in two of the most unequilibrated (unmetamorphosed) type LL3 ordinary chondrites, Bishunpur and Semarkona. Water released from bulk unseparated material by pyrolysis has δD values up to $\sim 3,000\%$ (SMOW) or in absolute terms a D/H ratio of $\sim 6 \times 10^{-4}$. Thus, one of the highest deuterium contents yet encountered for a Solar System species has been observed without considering a specific fraction of a sample. Either these meteorites contain a greater proportion of the D-rich phase first recognized in meteorite separates, or a small fraction having an even more extreme deuterium content resides within them.

Variations in deuterium abundance are usually small and are reported relative to standard mean ocean water (SMOW):

$$\delta D = \left[\frac{(D/H)_{\text{sample}} - (D/H)_{\text{SMOW}}}{(D/H)_{\text{SMOW}}} \right] \times 1,000\%$$

Hydrogen in terrestrial waters and other materials has a range of isotopic compositions falling within several hundred per mil of the absolute D/H ratio $(1.56 \times 10^{-4})^2$ of SMOW².

Before 1977 only two studies of meteorite D/H ratios had been attempted. Both concerned carbonaceous chondrites and had given inauspicious results in terms of anomalous compositions. Boato³ recognized adsorbed or terrestrially exchanged H₂O which could be removed by heating at temperatures up to 200 °C and more significantly, he found hydrogen (probably from hydrated silicates and organic matter) released at higher temperatures was enriched (up to 300%) in D relative to SMOW. Briggs⁴ studied D/H ratios of solvent extractable organic materials; again enrichments up to 200% were encountered which led him to argue for an extraterrestrial origin for the compounds investigated.

More recently Robert *et al.*⁵ have studied ordinary chondrites in an attempt to differentiate between water from hydrated minerals and from the decomposition of organics. All the samples considered, with the exception of Chainpur, an LL3 chondrite, were within the terrestrial range. Subsequent investigations^{6,7} with material separated from Chainpur suggest the existence of a phase(s), labile between 450 and 750 °C and occurring with or within chondrules, which has a δD value of +2,000 to +4,400%; that is, containing a factor of ~ 5 times more deuterium than SMOW, an enormous enrichment by terrestrial standards. Kolodny *et al.*⁸ reappraised the organic hydrogen in carbonaceous chondrites and postulated the presence of organic material in the meteorite Renazzo with a δD of +1,600% relative to SMOW. This effort was criticized^{9,10} on

the grounds that the low-temperature oxygen plasma used to remove the organics selectively, in fact removed more hydrogen than could be accounted for by the original organic content; the material balance calculations used by Kolodny *et al.*⁸ did not allow for this. Robert *et al.*⁹ and Robert and Epstein¹¹ investigated extractable and polymeric organics from the Murray meteorite and measured directly D enrichments approaching +1,000‰ in this specimen, whereas Smith and Rigby¹⁰ obtained δD values of +450‰ for acid insoluble residues from several carbonaceous chondrites.

While carbonaceous chondrites have been extensively studied for their hydrogen isotopic contents, primitive unequilibrated ordinary chondrites, except for Chainpur, have largely been ignored. It is now accepted^{12,13} that class 3 unequilibrated meteorites may be further subdivided according to the degree of unequilibration¹⁴; the number of subdivisions may be up to 10 (ref. 12). Although there is some disagreement as to whether Chainpur is one of the most unequilibrated examples of the class, it is accepted that three LL3 samples, Bishunpur, Krymka and Semarkona may be considered the most primitive^{12,13}. If the anomalously heavy hydrogen in Chainpur is a pre-Solar System remnant, it might also be found in these particular specimens.

Preliminary experiments on material from close to the fusion crust of a small sample from Bishunpur showed that water could be extracted with a δD value of between +2,489 and +3,109‰ SMOW (ref. 15). These results are for water released above 200 °C (to avoid terrestrial contamination^{3,7}) and below 750 °C, the temperature by which most water has been released from Chainpur⁷. Comparable sized samples of local meteoric water transferred through the same apparatus and distilled water used for cooling when cutting the meteorite gave δD values typical of the latitude of the laboratory (−44 and −48‰ SMOW respectively).

The latter experiments are not a true procedural check because temperatures up to 750 °C were not used. Because the Cambridge laboratory uses deuterium for various labelling purposes, we aimed to demonstrate the validity of the preliminary results. Our experimental procedure is: for each water extraction we used a new quartz reaction vessel prebaked to >1,000 °C overnight on a line run at 200 °C. During water collection this vessel is valved off from the pumping system. Before sealing the extracted waters in a quartz takeoff finger (trapped by liquid N₂ for 2 h) for transfer to East Kilbride for isotopic measurements, non-condensable gases are pumped away by opening the valve to the high vacuum line for ~10 s. There is no possibility of back trapping of water from the vacuum system during this pump-out because of the presence of a protecting liquid N₂ trap immediately next to the valve on the system side. During our experiments aliquots of a terrestrial biotite were processed alternately with the samples not as a measure of reproducibility, but as a contamination check.

All water samples were converted to hydrogen using the conventional hot uranium technique¹⁶. Isotopic analysis of hydrogen was performed on a VG Micromass 602 mass spectrometer using a reference of $\delta D = -50$ ‰ relative to SMOW, produced from doubly distilled water and calibrated against the international standards V-SMOW and V-SLAP. The memory correction associated with reducing waters widely separated in δD was estimated from V-SMOW and V-SLAP analyses to be 1.5‰ and this has been applied to all meteorite data.

All our D/H data from unequilibrated ordinary chondrites are listed in Table 1. Bishunpur and Semarkona release water extremely enriched in deuterium. The biotite blanks run alternately with the samples indicate the results obtained are neither an analytical artefact nor the manifestation of deuterium contamination introduced in the Cambridge laboratory. The spread in biotite δD values is considerably greater than that of replicate total hydrogen extraction (± 2 ‰, 1σ) and is attributable to incomplete yields for biotite using the experimental technique devised for meteorites.

Sample inhomogeneity is common on all scales in the least equilibrated ordinary chondrites¹⁴ and may explain the dis-

Table 1 D/H data from unequilibrated ordinary chondrites

Sample	Wt (mg)	wt % H ₂ O [§]	δD ‰ (SMOW)
Bishunpur 1*	~200	ND	+2,489
Bishunpur 2*	81.9	ND	+3,109
Biotite	21.0	ND	−35.3
Krymka†	98.7	ND	+54
Biotite	20.5	ND	−67.8
Semarkona‡	31.1	0.34	+2,904
Biotite	31.7	0.74	−62.6
Bishunpur 3†	31.2	0.29	+1,101
Biotite	30.4	0.71	−77.3
Biotite	36.4	0.79	−21.0
Weston†	37.7	0.23	+16.1
Biotite	30.7	0.60	−13.8

* Supplied by Dr S. O. Agrell (University of Cambridge) run with alternating tap water samples¹⁵.

† Supplied by Dr R. Hutchison (British Museum).

‡ Supplied by Dr R. Clarke (Smithsonian Institution).

§ Measured manometrically as H₂.

|| Corrected for memory contribution of 1.5‰, from hydrogen $\delta D \sim -40$ ‰. ND, not determined.

crepancy between the Bishunpur 3 result and those of the preliminary study¹⁵; Bishunpur 3 was obtained from an alternative source to the other two aliquots and the amount analysed was substantially less. The apparent inhomogeneity of Bishunpur may be significant and might indicate a sporadic occurrence of a minor very D enriched phase. We cannot explain the Krymka result which seems to suggest that the occurrence of anomalously heavy hydrogen is not simply a question of degree of unequilibration, although very inhomogeneous distribution of D rich phases cannot be ruled out. Water extracted from dark portions of the nominally H4 chondrite Weston, which has H3 type inclusions¹⁷ and water present in veins of iddingsite¹⁸, does not exhibit an extreme D content. However, Weston has a complement of solar wind gases¹⁹ and any hydrogen present from the solar wind might be expected^{20,21} to have a D/H of $< 3 \times 10^{-6}$ possibly counterbalancing a component enriched in D. The δD value obtained for Weston in this study (+16‰) is greater than that of −100‰ reported by Robert *et al.*⁷.

Our results are entirely for bulk unseparated meteorites. No attempt has been made to preconcentrate or selectively decompose the host phase of the water analysed. Alternative explanations for the Bishunpur and Semarkona data are either: (1) D enrichment is ubiquitous and all the hydrogen turning up in the water extracted has an isotope composition approaching that observed in certain Chainpur separates or (2) somewhere in these very unequilibrated chondrites is a phase of exceptionally high D content. In the case of Chainpur, stepwise heating and selection resulted in a δD increase from +200‰ for bulk material⁵ to +4,400‰ for the 450–750 °C temperature cut from several chondrules⁷. If explanation (2) is correct for Bishunpur and Semarkona, a comparably successful future isolation study ought to reveal a truly extreme hydrogen isotopic composition. Either way, studies of the material separated from Bishunpur and Semarkona should help to identify the D-enriched host phases. A possible site for a D-rich phase surviving accretionary processes might be the opaque matrix reported by Huss *et al.*¹⁴ and thought to be the low temperature condensate of Larimer and Anders²².

The problem of D-enriched molecules in primitive meteorites has been extensively discussed elsewhere²³. It might have been easier to accept deuterium depletions as the protosolar nebula is thought²⁴ to have had a D/H ratio of 2.5×10^{-5} and unmetamorphosed meteorites could have been expected to have retained some original material. Geiss and Reeves²³ concluded ion–molecule reactions at low temperatures to be the most likely means of concentrating deuterium, and inferred that the deuterated species in Chainpur and carbonaceous chondrites were remnants of dark interstellar clouds as first suggested as a possibility by Kolodny *et al.*⁸. We must concur with this interpretation

as it is very unlikely that an elementary experiment such as ours has recognized the maximum deuterium enrichment in a Solar System material, and ion-molecule reactions seem to be the only feasible route to major fractionations. In dark clouds the greatest D enrichments (10^3 – 10^4 compared to SMOW) are observed for HCO, isocyanides and cyanides²⁵, the latter being enriched by exchange reactions with CH_2D^+ or H_2D^+ . The D enrichment in water in interstellar clouds is difficult to measure; Geiss and Reeves suggest a D content 50 times that of terrestrial water but recognize they are possibly an order of magnitude in error. If the heavy hydrogen in Bishunpur and Semarkona is confined to a specific fraction as seems to be the case in Chainpur⁷, and indeed exists as water, it is not inconceivable that a practically 'pure' sample of interstellar water has survived into the Solar System. It could be just as important to recognize that the heavy hydrogen is associated with some polymeric carbonaceous phase which condensed from deuterium enriched species. Unequilibrated ordinary chondrites have up to 0.5 wt% carbon²⁶, which as yet is substantially uncharacterized.

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Production of NO and N₂O by soil nitrifying bacteria

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The composition of the atmosphere is influenced both directly and indirectly by biological activity. Evidence is presented here to suggest that nitrification in soil is a potentially significant source of both NO and N₂O. Between 0.3 and 10% of the ammonium oxidized by cultures of the soil bacterium *Nitrosomonas europaea* is converted to these gases. The global source for NO associated with nitrification could be as large as 15×10^6 tonnes N yr⁻¹, with a source for N₂O of 5 – 10×10^6 tonnes N yr⁻¹. Nitric oxide has a key role in tropospheric chemistry, participating in a complex set of reactions regulating OH and O₃ (refs 1,2). Nitrous oxide is a dominant source of stratospheric NO (refs 3–5) and has a significant influence on climate⁶.

Table 1 Relative rates for NO, N₂O and NO₂⁻ production by cultures of *Nitrosomonas europaea*

Oxygen concentration (% v/v)	NO/N ₂ O (mol N/mol N)	N ₂ O/NO ₂ ⁻ (% mol N/mol N)	NO/NO ₂ ⁻ (% mol N/mol N)	No. of replicates
0.5	5.1 ± 3	0.9 ± 0.3*	3.8 ± 1.2	2
0.5		2.5 ± 0.1	—	3
1.0		0.9 ± 0.1	—	3
1.3	1.4†	—	1.25 ± 0.2	2
5.0		0.15 ± 0.02	—	3
10.0		0.16 ± 0.01	—	3
21.0		—	0.13 ± 0.1	2
21.0	1.15 ± 0.15	0.47 ± 0.13	0.51 ± 0.1	2

Ratios are given as mol % for N₂O and NO relative to NO₂⁻, but as mol/mol for NO relative to N₂O.

* Production of N₂O increased relative to NO₂⁻ in this run, from 0.5% initially to 1–2% at the end.

† Estimated from N₂O/NO₂⁻ at 1% O₂ and NO/NO₂⁻ at 1.3% O₂.

Enzyme preparations derived from nitrifying bacteria are known to produce trace quantities of NO and N₂O (refs 7,8) and release of these gases has also been observed from dense cultures⁹. Góreau *et al.*¹⁰ investigated rates for production of N₂O by marine nitrifying bacteria, grown under a range of oxygen concentrations from 0.5 to 20%. The yield for N₂O, defined as the ratio of N₂O produced relative to NH₄⁺ oxidized, was about 0.003 mol N N₂O per mol NH₄⁺ at O₂ concentrations above 2%. The yield rose sharply at lower concentrations of O₂, reaching 0.1 for an O₂ concentration of 0.5%. The investigation of Góreau *et al.*¹⁰ is extended here with studies of a soil nitrifier, *N. europaea*.

Cells from actively growing cultures were inoculated into 500-ml flasks containing 300 ml of aqueous growth medium¹⁰ with 25 mmol NH₄⁺. The initial density of cells was $\sim 10^6$ cm⁻³. The medium was stirred continuously, purged by a stream of gas consisting of N₂ and CO₂ (300 p.p.m.), with predetermined concentrations of O₂. Temperature was maintained at 25 °C, with pH buffered at 7.5.

Flushing time of the flasks was fixed in the range 4–10 min, sufficient to ensure equilibrium between liquid medium and gaseous headspace. Medium was sampled periodically and analysed for NO₂⁻ and cell numbers (see ref. 10). Concentrations of nitrous oxide and oxygen in the outflow were measured using gas chromatography with electron capture and thermal conductivity detection respectively¹¹. Nitric oxide was measured using a chemiluminescence detector, with procedures designed to eliminate interference from other gases¹². Production of N₂O was measured at O₂ levels of 20, 10, 5, 1 and 0.5%. Production of NO was determined for O₂ concentrations of 20, 1.3 and 0.5%.

Experimental results are presented in Fig. 1 and Table 1. Production rates for N₂O, NO and NO₂⁻ were closely parallel, as observed previously¹⁰ for NO₂⁻ and N₂O. Cultures produced N₂O and NO with uniform ratios relative to NO₂⁻ at O₂ levels above 1% (Fig. 1a, c). Replicate flasks gave consistent results, with variability largest at low O₂. The rate for evolution of NO correlated strongly with that for N₂O (see Fig. 1d). Measurements of cell numbers showed that cell division did not occur in cultures with O₂ equal to 20%, and production of NO₂⁻, NO and N₂O were constant with time in these experiments (see Fig. 1a). Active cell division occurred in all other experiments, with doubling times of 0.8 ± 0.2 days. Yields for NO and N₂O were constant relative to NO₂⁻, independent of whether or not cells were dividing. An exception occurred at 0.5% O₂, where the yield for N₂O increased with time even though the yield for NO remained constant.

Results for NO and N₂O are given in Table 1 at various oxygen concentrations. Yields for both gases rose markedly at O₂ concentrations below 2%. The ratio of NO production relative to N₂O also rose as O₂ decreased, with a median value of ~ 1.5 mol N NO per mol N N₂O.

Two experiments were performed which indicated a metabolic origin for the bulk of NO observed in the present work. First, sterile culture medium was spiked with NO_2^- to a concentration above that in the active cultures. A small transient pulse of NO was observed but emission terminated within minutes. Second, an actively growing culture of *N. europaea* was killed by addition of 1 ml saturated solution of a potent respiratory inhibitor, HgCl_2 . The NO signal declined promptly, falling within 50 min to 10% of the value observed before addition of HgCl_2 . Decay of NO followed the time course expected from flushing of the experimental apparatus, with negligible (<10%) abiotic production. The behaviour was similar to that observed earlier for N_2O (ref. 10).

The concentration, C (cm^{-3}), of NO or N_2O measured at the outlet of the apparatus may be written in the form

$$C = Pt/(1 + Lt)$$

where t is the flushing time (s), P is the production rate per unit

volume of the apparatus ($\text{cm}^{-3}\text{s}^{-1}$) and L is a loss frequency (s^{-1}) introduced to account for possible loss in the medium, in the head space or on the walls of the apparatus. Figure 2 shows a plot of CV/t , the mass flux from the apparatus, as a function of t (where V is the volume of the apparatus, $t = V/(\text{flow rate})$). This quantity should be independent of t for $Lt \ll 1$ and should vary as t^{-1} for $Lt \gg 1$. The results in Fig. 2 suggest that losses of NO are negligible in the present system, that $L^{-1} \gg 4$ min. Loss of N_2O is similarly small¹⁰.

Nitrification is thought to provide a major source for atmospheric N_2O (refs 10, 13, 14). The global source of N_2O , known from considerations of atmospheric chemistry¹⁵, is 10×10^6 tonnes N yr^{-1} . If we assume that this source is associated primarily with nitrification and adopt relative production rates indicated in Table 1, we may estimate that the corresponding source of NO could be as large as 15×10^6 tonnes N yr^{-1} . There may be sources of N_2O in addition to nitrification, for example, denitrification¹⁶ and lightning¹⁷. Our estimate for the

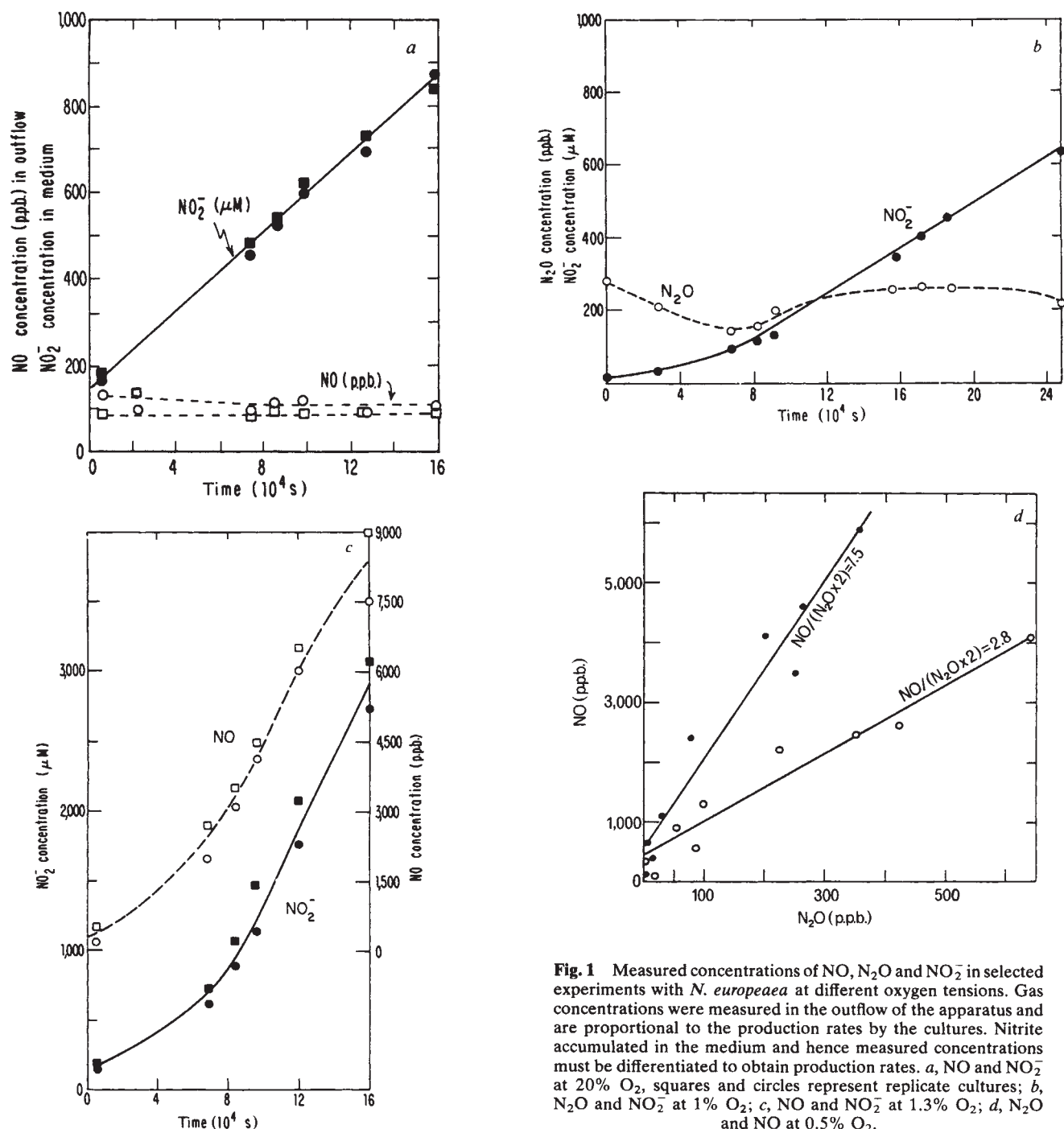


Fig. 1 Measured concentrations of NO, N_2O and NO_2^- in selected experiments with *N. europaea* at different oxygen tensions. Gas concentrations were measured in the outflow of the apparatus and are proportional to the production rates by the cultures. Nitrite accumulated in the medium and hence measured concentrations must be differentiated to obtain production rates. a, NO and NO_2^- at 20% O_2 ; squares and circles represent replicate cultures; b, N_2O and NO_2^- at 1% O_2 ; c, NO and NO_2^- at 1.3% O_2 ; d, N_2O and NO at 0.5% O_2 .

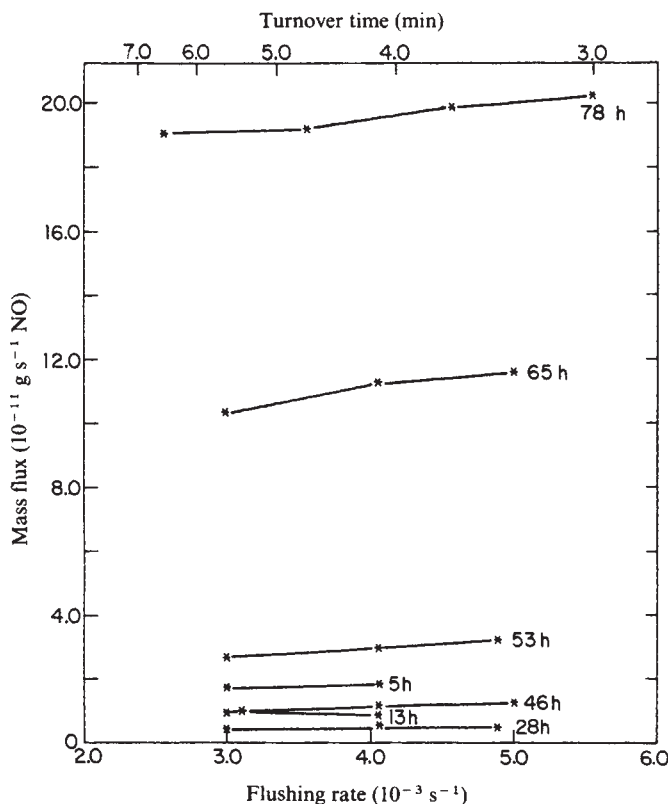


Fig. 2 The mass flux of NO (g NO s^{-1}) is plotted as a function of gas flow rate through the apparatus, at various times after inoculation. The flushing rate is defined as (flow rate)/(head space volume). For 0–46 h, purge gas contained 20% O_2 , the cells did not divide and NO was produced at low rate. Reduction of O_2 to 0.5% induced almost immediate increase in production of NO, NO_2 and bacterial cells. At both oxygen levels the mass flux seems to be independent of flow rate, implying negligible loss of NO in the apparatus (see text).

nitrification source of NO would be lowered accordingly. Also, NO could be removed by heterogeneous processes before release to the atmosphere (see ref. 12).

Galbally and Roy¹⁸ observed fluxes of NO with an average value of $2 \times 10^{-12} \text{ kg N m}^{-2} \text{ s}^{-1}$ from soils in Australia. They estimated a corresponding global source of 10×10^6 tonnes N yr^{-1} , similar to the value derived here. Our data are consistent with the view that nitrifying bacteria could produce a substantial fraction of the NO observed by Galbally and Roy¹⁸, although there could also be production by denitrification¹⁹ or by abiotic reactions involving NO_2^- (refs 20,21).

The source of NO derived here is similar in magnitude to production by combustion^{2,22}, photooxidation of NH_3 (ref. 23), decomposition of stratospheric N_2O (refs 15,22) and lightning (ref. 24). The lifetime of fixed nitrogen species in the atmosphere is rather brief^{2,25}. It follows that nitrification in soils could exercise a major influence on the NO_x budget of the troposphere, with particular significance for regions remote from human activity.

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Structure sensitivity in the iron single-crystal catalysed synthesis of ammonia

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There have been many investigations of the kinetics of the iron-catalysed synthesis of ammonia and the details of the mechanism of its reaction^{1–3}. However, we report here the first direct and, therefore, unambiguous measurement of the surface structure sensitivity of the iron-catalysed ammonia synthesis reaction using the (111), (100) and (110) crystal faces of iron and operating at pressures approaching those used industrially (20 atmospheres). The Fe(111) face was found to be the most active, the activity ratio being 418:25:1 for the Fe(111), Fe(100) and Fe(110) planes. We have also observed the formation of a strongly bound nitrogen species after the reaction, in the presence of which ammonia continues to be produced.

The apparatus⁴ consisted of a stainless steel ultrahigh vacuum chamber equipped with a four-grid retarding field analyser for Auger and low-energy electron diffraction measurements of the surface composition and structure, a residual gas analyser, an ion gun for specimen cleaning and a high-pressure cell connected to an external loop. Reactant and product gases at a total pressure of 20 atmospheres were continuously circulated over the heated iron crystal specimen by a positive displacement pump; samples, periodically withdrawn using a gas chromatograph type sampling valve, were passed by a nitrogen carrier through a photoionization detector to determine the amount of ammonia in the loop. Ammonia was the only species present which could be detected by the photoionization detector obviating the need for a separating column.

Circular iron specimens with crystal faces (111), (110) and (100) were spark-cut from a high-purity (4N) single-crystal rod and diamond polished, the final dimensions being ~6 mm diameter and 1 mm thickness. Sulphur and carbon were found to be the principal contaminants after this procedure, but could be removed by several days of heating at 800°C in 1 atmosphere of hydrogen, followed by many cycles of argon ion bombardment and annealing at 700 °C. To minimize the catalysis occurring on ill-defined iron surfaces, platinum foil was spot-welded to the rough edges of the specimens.

Results of measurement of structure sensitivity are shown in Table 1 from which it can be seen that the (111) surface of iron catalyses the synthesis of ammonia from hydrogen and nitrogen at least 418 times faster than the ordered (110) surface and 16 times faster than the ordered (100) surface. Our result is consistent with the low pressure chemisorption studies of Ertl and co-workers² where it was reported that the rate of dissociative chemisorption of nitrogen is greatest on the (111) plane of iron

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Table 1 Surface structure sensitivity of ammonia synthesis

Catalyst surface	Area (cm ²)	Rate* (mols NH ₃ cm ⁻² s ⁻¹)
Fe(111)	0.63	4.6×10^{-8}
Fe(100), ordered	0.80	2.8×10^{-9}
Fe(110), ordered	0.57	0†
Fe(100), disordered‡	0.80	4.5×10^{-9}
Fe(110), disordered‡	0.57	9.7×10^{-10}

* Total pressure, 20 atm.; $P_{H_2}/P_{N_2} = 3/1$; temperature = 798 K.

† Detection limit $\sim 1.1 \times 10^{10}$ mol NH₃ cm⁻² s⁻¹.

‡ Argon ion bombardment (1.5×10^{-5}) A cm⁻² for 10 min at 500 V.

and least on the (110) plane, and also with previous suggestions^{1-3,5} that this step is rate-determining in the ammonia synthesis reaction.

The (111) plane of iron contains both seven-coordinated (C₇) and four-coordinated (C₄) atoms, whereas the (110) and (100) planes contain only six-coordinated (C₆) and C₄ atoms, respectively. The large variation in rates of ammonia synthesis on the various planes (Table 1) and the anomalously high catalytic activity that we have also detected on the (110) and (100) planes when deliberately disordered by ion bombardment, support the proposals of Brill⁶ and others^{3,7} regarding the importance of clusters and C₇ iron atoms as components of the active site on the catalyst surface.

Although bulk nitrides of iron are thermodynamically unstable in the conditions of these experiments, we have observed the existence of a long-lived, strongly-bound nitrogen species after the synthesis reaction on both Fe(111) and Fe(100) crystal faces in the presence of which ammonia continued to be produced at an undiminished rate. The catalytic significance of this surprising observation has yet to be determined.

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An oceanic carbonatite volcano on Santiago, Cape Verde Islands

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Carbonatite volcanoes are rare, the only active one being at Oldoinyo Lengai in northern Tanzania¹ although a few dead volcanic centres with recognizable carbonatitic extrusive materials are known elsewhere in Africa²⁻⁶ and one in Germany⁷. During an expedition in 1980 to the Cape Verde Islands, in the Central Atlantic Ocean 500 km west of Senegal, the eroded remains of a carbonatite volcano were discovered on the island of Santiago just north of Tarrafal at Arruela (Fig. 1). This, the first example of an oceanic carbonatitic volcanic pyroclastic structure, shows typical carbonatite mineralogy and geochemistry, and demonstrates that carbonatite magmas can be generated in the oceanic lithosphere as well as in the continents.

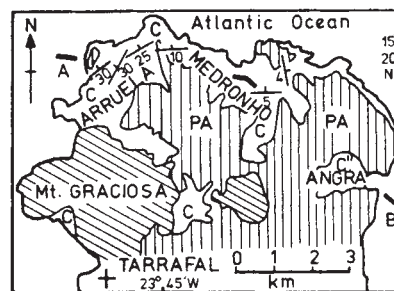


Fig. 1 Geological sketch map of northern Santiago (after Serralheiro⁹). ▨, Phonolite (youngest lavas); ▩, Pliocene plateau basalts of Pico da Antonia Formation (PA); ▤, intrusive, pyroclastic and epiclastic carbonatitic lithic tuffs. C' is explained in Fig. 2.

An oceanic character for the Cape Verde Islands region seems the most probable, although the possibility of a fragment of continental crust existing in the area cannot be totally discounted. However, the position of these volcanic islands some 400 km west of the westernmost parts of the West African continental shelf astride the Mesozoic oceanic geomagnetic anomalies^{8,9}, the mid-oceanic ridge basalt geochemical character of the pillow basalts of the region¹⁰ and the fact that the islands have apparently grown from the oceanic floor, leave little doubt as to their oceanic setting.

Carbonatitic magmatism has been said to characterize intraplate continental crust⁵, but this discovery of a carbonatite volcano and therefore of undoubted carbonatite magmatism demonstrates that such magmas can form in oceanic lithosphere. The intrusive carbonatitic equivalents, together with ijolites, on most of the other Cape Verde Islands (and some of the Canary Islands) suggest that intraplate oceanic carbonatitic magmatism is not rare; in fact it is commonplace in all oceanic islands with long geological histories so far investigated.

Only the basal wreck of the former pyroclastic cone on Santiago remains (Fig. 2), and it comprises carbonatitic and nephelinitic subaerial tuffs pierced by carbonatite and nephelinite dykes and veins. In every petrological feature, apart from the absence of natrocarbonatite lava, the structure and petrology are exactly similar to those at Oldoinyo Lengai volcano. The greater part of the cone seems to have been planed off by erosion, and much of the material redeposited as shallow marine epiclastic carbonatitic conglomerates, siltstones and calcilutites. These are covered by bioclastic calcarenites and plateau basalts.

Beneath the erosion level, exposures in the western sea-cliffs reveal great thicknesses of massive pale-coloured carbonate-cemented agglomerates, ashes and tuffs which carry an assortment of coarse-grained, fine, dark, light-coloured, big (1m) and small blocks. Elsewhere the rocks are better bedded with occasional large-scale low-angled cross-bedding, generally dipping seawards. They and the associated agglomerates are calcareous, and all abound with biotite flakes and lithic fragments including apatite-sövite, ijolite, apatite-pyroxenite and olivine-poor nephelinite.

The unique carbonatitic character of the sub-aerial ochre-coloured tuffs which make up the bulk of this former volcanic structure, is not immediately apparent in the field, and had not been recognized by previous workers^{11,12}. The microscope reveals that the tuffs are 95% or more of rounded grains of calcite crystallites, each grain usually having a brown-stained core. This texture is similar to that seen in carbonatitic tuffs in East Africa. Some horizons include lapillistone containing spherical lava tears with quench textures identical to those described recently from Kaiserstuhl⁷. Interleaved with the carbonatitic tuffs and agglomerates are large masses of pale grey-green unbedded clays up to 5 m thick. As the clays carry abundant ijolite and nephelinite clasts, these clays are interpreted as altered nephelinitic tuffs and ashes. The assemblage is identical to that at Oldoinyo Lengai¹, and it all points to a mainly pyroclastic mechanism of eruption with much of the lava being emitted as droplets perhaps during *nuée ardente* eruptions.

Fig. 2 Cross-section along line A-B in Fig. 1 showing the eroded remains of the carbonatitic and pyroclastic volcanic cone (CA) penetrated by carbonatite adventive plugs (C') and dykes, and unconformably (shown schematically) overlain by carbonatitic epiclastic beds (CB) and calcarenites (dotted); above are Pico da Antonia basalts (PA). At Angra is a carbonatite-ijolite-syenite-breccia plug (C'') which intrudes basalt pillow lavas (P), and is overlain unconformably by PA basalts.



Penetrating the tuffs are a great variety of dykes and plugs. Several tuff dykes, 0.1–3.0 m wide, carry abundant ijolite fragments, some as cored autoliths with nephelinitic coatings. Other dykes which run in all directions are often only 0.2–0.5 m wide and include olivine-poor nephelinites (with nepheline, aegirine-augite, melanite-garnet, perovskite, apatite, analcime and some phlogopite) and carbonatites. One carbonatite dyke 0.2 m wide made up entirely of carbonate revealed, under the microscope, discontinuous pieces of convoluted flow texture of deformed ovoids, and appears to represent a flowed and fragmented carbonatitic pumice which must have been emplaced in a fissure not far below a point of extrusion.

Intrusive plugs, one up to 15 m diameter¹², of flow-banded dolomitic carbonatite brecciate their way through the bedded tuffs. Also penetrating the tuffs are vertical, roughly circular haematitic patches 1–3 m across, apparently representing paths of fumarolic fluids passing upwards through the ochreous tuffs.

An ⁸⁷Sr/⁸⁶Sr determination of the Arruela plug carbonatite (at the Université de Clermont-Ferrand) gave 0.7040 which is close to earlier determinations of other Cape Verde and Canary Island carbonatites^{13,14}. This confirms the igneous character of these carbonatite plugs. Preliminary X-ray fluorescence analysis (H. Furnes at Bergen University) for the trace element content of the carbonatites (Table 1) shows high contents of the incompatible elements which is typical of true carbonatites⁶. They are further noteworthy for the unusually high Zn content and the strong rare-earth enrichment in the intrusive carbonatites.

A period of sub-aerial followed by marine erosion seems to have decapitated this pyroclastic volcano, leaving it buried beneath 10–100 m of epiclastic sediments largely composed of detritus derived from the destruction of the volcano. Conglomerates and grits fill hollows in the underlying tuffs. The dips are variable and usually low (0°–20°) except where now seen in some slipped sections along the cliffs. Some beds are well stratified fine siltstones, some with graded bedding, others with cross-lamination. There are moulds of large marine gastropods. In further contrast to the volcanic tuffs below, these beds are rarely cut by dykes and then only by basaltic ones, and are quite well sorted. Clasts of ijolite, pyroxenite and nephelinite up to 0.1 m across are common and biotite flakes abound. Under the microscope these epiclastic siltstones and grits are seen to be composed of abundant grains of carbonatite, recognizably the

same as those in the underlying tuffs, and with detrital apatite, aegirine, garnet, perovskite and chert.

In places along the erosional unconformity with the overlying Pico da Antonia Formation alkali basalts, these lenticular epiclastic sediments are strongly reddened. There is also a concentration of ijolite, nephelinite, basalt and red carbonatite clasts in the conglomerate marking this unconformity. In the Ponta Moreia area, marine bioclastic calcarenites, a few metres thick and containing oncolites, usually lie immediately under the basalts.

It is estimated that the exposed area of the carbonatitic pyroclastic volcano remnant exceeds 10 km², and any envisaged reconstruction of a volcano on this site produces a structure very similar to that at Oldoinyo Lengai in northern Tanzania¹.

The main conduit of the volcano is unknown. The vent might be sited centrally in the northern part of Santiago and would then be hidden beneath the later basalt and phonolite lavas. But it is more likely that the intrusive ijolites, carbonatites and breccias at Angra, presently standing nearly 200 m above sea level and penetrating old pillow lavas, mark the site of the vent-plug. The age of the volcano is not known except that it is older than the Pico da Antonia basalts which previous workers estimate are Pliocene–Miocene¹². K–Ar dating¹⁵ gave 4–5 Myr for the basalts, and 8.5 and 9.8 Myr for intrusive carbonatites elsewhere in Santiago.

Carbonatitic volcanism must therefore no longer be considered as restricted to continental regions. This oceanic intraplate occurrence demonstrates that carbonatite magma can form in suboceanic as well as subcontinental lithospheric mantle. A further implication would seem to be that whilst small ocean islands such as St Helena represent small developments of alkaline magmatism, the bigger archipelagos such as the Cape Verdes seem to be the result of more extensive alkaline magmatism which can produce more extreme alkaline compositions. This probably reflects greater mantle metasomatism in the source regions. Mantle heterogeneity has been widely advocated recently¹⁶, but such extremes beneath oceans have not been suggested before.

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Table 1 Trace elements in Cape Verde carbonatitic rocks, preliminary results in p.p.m.

	1	2	3	4	5	6
V	136	140	200	89	435	114
Zn	240	304	245	309	617	758
Rb	6	7	7	10	14	13
Sr	490	968	1,176	6,210	4,500	4,320
Y	70	72	102	37	205	137
Nb	72	41	427	14	195	43
La	683	900	1,427	2,790	5,850	8,040
Ce	921	1,175	2,028	3,373	6,913	10,043
Nd	299	358	862	914	2,056	2,791

Columns 1 and 2: dolomitic carbonatitic intrusive tuffs of the Arruela plug. Column 3: carbonatitic tuff dyke cutting tuffs near the Arruela plug. Column 4: apatite-carbonatite, recrystallized block in carbonatitic breccia at Angra. Column 5: metabasalt veined and impregnated by carbonatite, Angra. Column 6: metabasalt metasomatized by carbonatite, Angra.

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Platinum and palladium minerals in upper mantle-derived lherzolites

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Spinel lherzolite xenoliths in basanites from the Newer Volcanics of Western Victoria, Australia contain sulphide inclusions along grain boundaries and within fractures in the silicates. The inclusions are complex intergrowths of pentlandite, pyrrhotite and chalcopyrite formed by the breakdown of Fe-Ni monosulphide solid solutions. During this process discrete grains of a platinum sulphide and a palladium stannide were exsolved from the pentlandite. We show here that this is the first evidence for sulphide fluids rich in platinum group elements in the upper mantle.

During an investigation¹ of the distribution of Au, Pd and Ir in mantle-derived spinel lherzolites from basanites of the Newer Volcanics of Western Victoria, Australia², it was discovered that the bulk (60–80%) of these metals are not held in solid solution in any of the major silicate or oxide phases but are concentrated in the intergranular regions¹. The intergranular phases consist of serpentines, magnetite, goethite and sulphides which are located along grain boundaries and within fractures in the silicates. Textural evidence indicates that the sulphides have been introduced into the xenoliths along these microfractures. The sulphides were not introduced into the xenoliths during transport as the host basanite lacks such materials, is undersaturated in sulphur and is poor in Au, Ir, Pd¹. We consider that the sulphides were added to the xenoliths in the mantle and represent an immiscible sulphide melt formed during a partial melting episode before that which generated the host lava which merely transports the xenoliths to the Earth's surface¹. In view of the chalcophilic properties of the platinum group elements³, it was proposed that these sulphides might be the major host of the intergranular platinum group elements¹. To investigate this

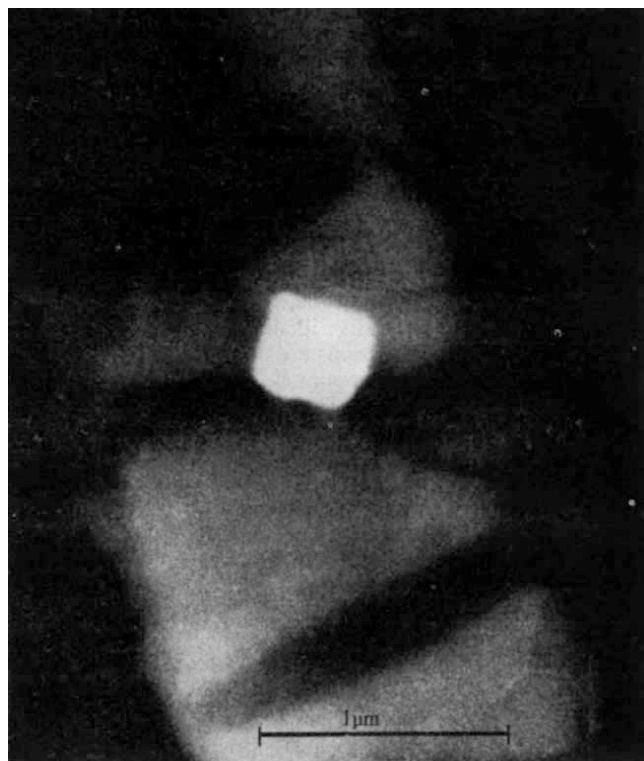


Fig. 2 Back-scattered electron photograph obtained using a Robinson detector coupled to a scanning electron microscope. The euhedral phase showing strong atomic number contrast (white) is platinum sulphide set in a pentlandite host (grey). Black areas are cleavages or fractures.

hypothesis we examined the sulphides using scanning electron microscopy and discovered discrete grains of platinum group minerals.

Figure 1a shows a typical ovoid sulphide inclusion and illustrates the complexity of the sulphide intergrowths. The bulk of the globule consists of pyrrhotite with exsolved flames of pentlandite, this assemblage being mantled by chalcopyrite containing exsolved mackinawite. The chalcopyrite is replaced along cleavages by magnetite.

Figure 1b shows another sulphide globule consisting of pentlandite which has been replaced by magnetite. Within this

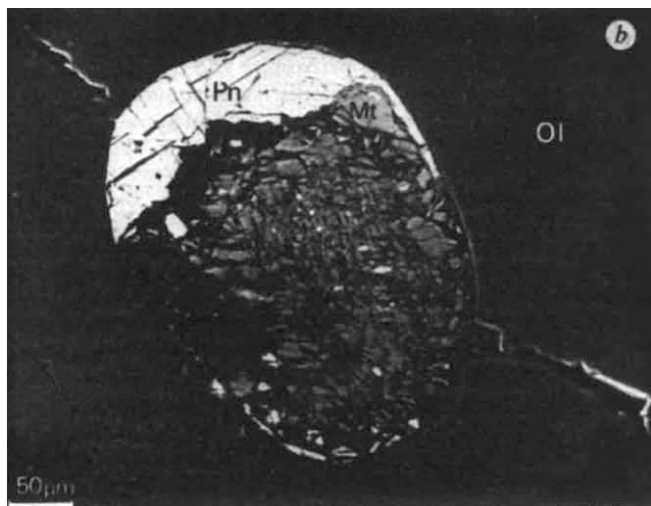
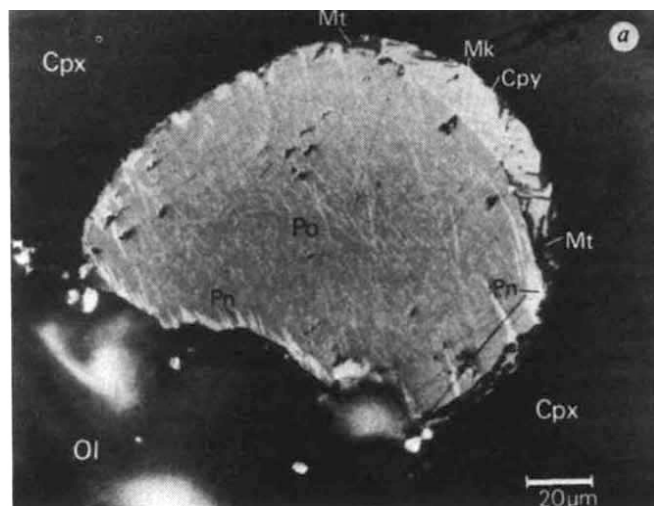


Fig. 1 *a*, Composite sulphide globule at the contact between olivine (Ol) and clinopyroxene (Cpx) crystals. The main mass of the globule consists of pyrrhotite (Po) containing exsolved pentlandite (Pn). This is mantled by chalcopyrite (Cpy). Thin lamellae of mackinawite (Mk) occur within the chalcopyrite which is also replaced along cleavages by magnetite (Mt). This photomicrograph illustrates the incipient replacement of sulphides by oxides. Reflected light photograph of polished thin section. *b*, Sulphide globule located within a fracture in olivine consisting of pentlandite and magnetite. This was originally an entire sulphide globule similar to that shown in *a*. The black areas mark plucked sulphide or oxide. Reflected light photograph of polished section.

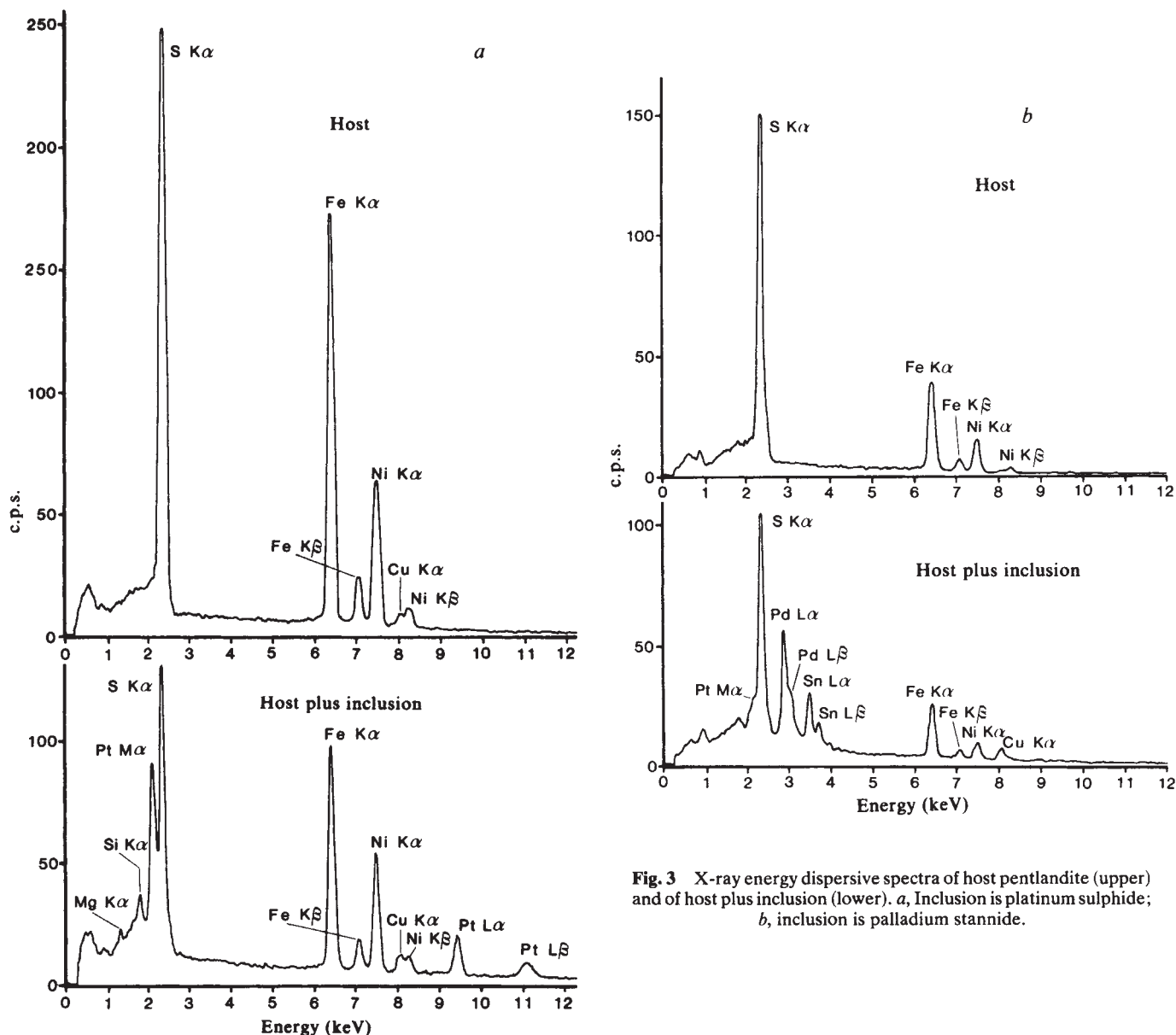


Fig. 3 X-ray energy dispersive spectra of host pentlandite (upper) and of host plus inclusion (lower). *a*, Inclusion is platinum sulphide; *b*, inclusion is palladium stannide.

globule we located the discrete Pt-mineral described below. The original mineralogy of the globule before magnetite replacement was probably similar to that of the inclusion shown in Fig. 1*a*.

Platinum group minerals were located using a Robinson back-scattered electron detector coupled to a scanning electron microscope⁴. This type of imaging enhances the atomic number contrast for back-scattered electrons from matrices of differing average atomic number and is ideally suited for the detection of platinum group minerals of high atomic number in a Fe-Ni-sulphide matrix. Figure 2 illustrates the excellent atomic number contrast exhibited between a Pt-mineral and its pentlandite host.

The inclusion is too small (0.5 μm) to extract for X-ray diffraction identification and for quantitative *in situ* electron microprobe analysis, as the excitation area produced by the electron beam is larger than the inclusion, resulting in excitation of the host. Figure 3*a* thus shows X-ray spectra of the pentlandite host and of the host plus inclusion to demonstrate that the inclusion is a discrete phase. The spectrum of the host plus inclusion contains strong Pt X-ray lines which are lacking in the host alone. The presence of minor peaks attributable to Mg and Si indicates fluorescence of intergranular serpentine or of the olivine which hosts the sulphide inclusion. The mineral lacks As, Sb, Bi, Pd and Sn, any S present is masked by the S peaks of the

pentlandite. From this qualitative analysis we believe the inclusion to be a platinum sulphide, probably cooperite (PtS).

Examination of other sulphide globules resulted in the discovery of several anhedral grains of a palladium mineral in a pentlandite host. Figure 3*b* shows X-ray spectra for the host and the inclusion plus host. The presence of strong X-ray lines of Pd and Sn together with only a weak Pt line and the absence of As, Sb, Bi indicates that the mineral is a palladium stannide. Unfortunately, we were not able to analyse this phase quantitatively. The mineral could possibly be paolovite (Pd_2Sn), atokite (Pd_3Sn) or stannopalladinite ($\alpha\text{-Pd}_3\text{Sn}_2$).

Sulphide globules containing complex Cu-Ni-Fe sulphide intergrowths similar to those described above have been noted to occur in spinel lherzolites and eclogites⁵⁻⁷. However, to our knowledge this is the first occurrence of discrete Pt and Pd minerals in such an upper mantle xenolithic paragenesis.

Studies of magmatic nickel-platinum group element ore deposits such as those associated with the Sudbury and Bushveld intrusions coupled with experimental studies of Cu-Ni-Fe sulphide phase equilibria have demonstrated that the precursors to the pentlandite-pyrrhotite-chalcopyrite intergrowths are Fe-Ni-monosulphide solid solutions which crystallized from a sulphide liquid initially formed by liquid immiscibility from a sulphur saturated basic silicate melt^{8,9}. The originally homogeneous monosulphide undergoes a variety of subsolidus

breakdown reactions as it cools from magmatic temperatures ($\sim 1,000^\circ\text{C}$). For example, pentlandite only becomes stable below 610°C and the pentlandite-pyrrhotite intergrowths probably develop below 300°C (ref. 6). Platinum group minerals in such parageneses occur as discrete grains in sulphide host and exhibit textural relations similar to those observed in the spinel lherzolites. Cabri and Laflamme¹⁰ proposed that most of the platinum group minerals in such ores must also have originated by sub-solidus exsolution from a platinum group element-rich sulphide. Accordingly we believe that the discrete Pt and Pd minerals noted above have exsolved from pentlandite at low temperatures, the pentlandite itself having previously formed by the low temperature breakdown of Fe-Ni monosulphide solid solution. The initial sulphide liquid was introduced into the lherzolite within the mantle and in those conditions would have crystallized to a homogeneous platinum group element-rich monosulphide solid solution. Sub solidus breakdown probably occurred after incorporation into the transporting magma during cooling of the lava subsequent to eruption.

Speculation concerning the source of the sulphide liquids is beyond the scope of this note, but the above observations demonstrate that platinum group element-rich sulphide liquids are present in the upper mantle. This conclusion has important consequences with regard to the geochemistry of the platinum metals in general and in particular to their distribution between the Earth's core and mantle and to the possibilities of the addition of magmatic sulphide liquids to lherzolite within the upper mantle.

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Cross-polarization ^{13}C -NMR spectroscopy with 'magic angle' spinning characterizes organic matter in whole soils

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The limitations of ^{13}C cross-polarization (CP) NMR spectroscopy with 'magic angle' spinning to characterize the organic matter in whole soils have recently been discussed¹: resonances from rotor materials and from spinning sidebands prevented a complete analysis of the types and forms of carbon in whole soils. We now report experiments in which these problems have been overcome. The results show that carboxylic, aromatic, acetal, alkoxy and alkyl carbon are present in soils and they allow approximate estimates of various carbon types to be made without previous extraction from the soil.

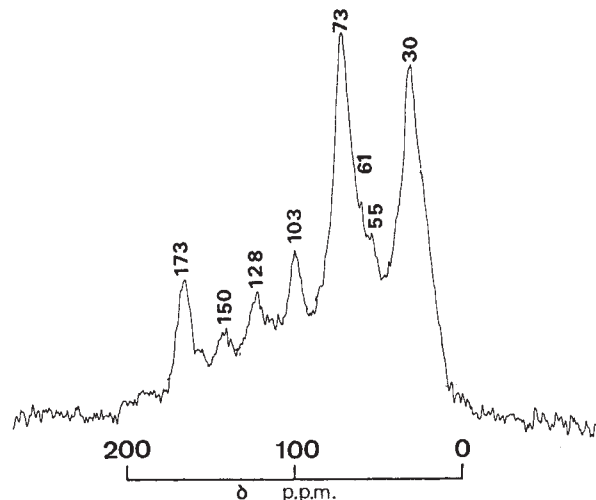


Fig. 1 CP-MASS spectrum of Mapourika soil. Spectrum was obtained with a contact time of 1 ms and a recycle time of 0.3 s; 10^5 transients were collected.

There have been no adequate techniques for characterizing soil organic compounds in the solid state. Moreover, the limited solubility of these materials, even in strongly alkaline media, makes studies on extractable material of limited applicability. CP- ^{13}C -NMR spectroscopy could provide qualitative information about the fraction of carbon which is aromatic, the aromaticity (f_a) of whole soils², but detailed information on the chemical structure of *in situ* organic matter is hidden because the linewidths of CP- ^{13}C -NMR signals in solids are of the order of 200 p.p.m. due to chemical shift anisotropy. Spinning the sample at an angle of $54^\circ 44'$ to the applied field (the 'magic angle' sample spinning technique, MASS) reduces linewidths to a magnitude comparable with that obtained for species in solution. Unfortunately, the low carbon content of soils precludes the use of conventional rotor materials (usually deuteropoly-methylmethacrylate) for obtaining spectra of whole soils^{1,3}. We have used a rotor consisting of a barrel of boron nitride with a seat of Kel F (K.W.Z., unpublished work), and sufficient spinning speeds (~ 3.5 kHz at 25.1 MHz ^{13}C frequency) to remove sidebands. Some characteristics of the soils used are given in Table 1 (more detailed information is available elsewhere⁴⁻⁷). Five soils have been investigated in this initial study, two young soils (age < 400 yr) and three older soils (age > 11,000 yr). Two

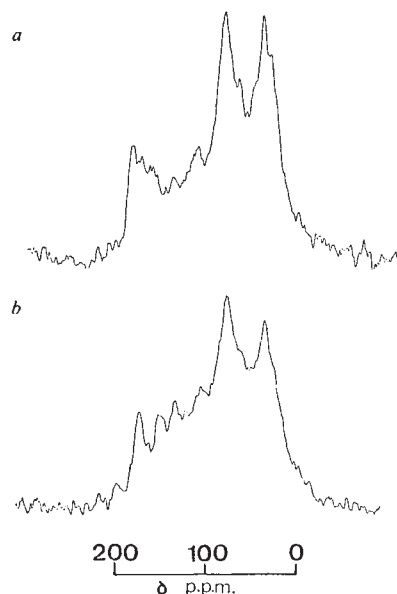


Fig. 2 CP-MASS spectra of a, Franz X and b, Ikamatu soil. Spectra were collected in similar conditions as Mapourika soil.

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Table 1 Characteristics of soils

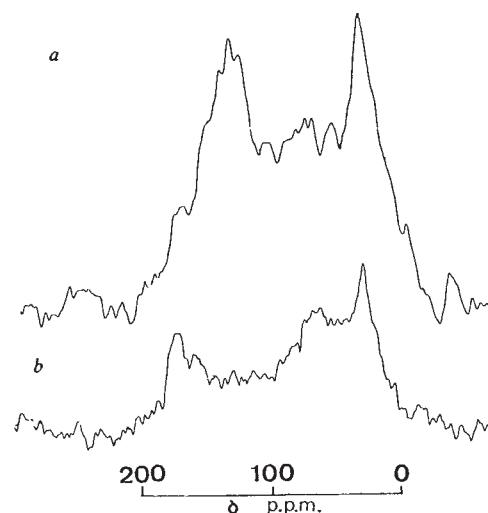
Soil	Horizon	Age (yr)	pH (1:2.5 with H ₂ O)	C (%)	Parent material	New Zealand classification
Hokitika	A	300	5.5	2.8	Glacial gravels	Recent
Ikamatu	A	14,000	4.6	9.8	Glacial gravels	Lowland yellow brown earth
Mapourika	A	12,000	4.2	7.9	Glacial moraines	Gley podzol
Franz X	A	350	5.2	6.6	Glacial moraines	Gley podzol
Tirau	A	—	5.7	10.5	Rhyolitic ash	Central yellow brown loam

of the soils (Hokitika and Ikamatu) form part of the Reefton chronosequence and two others (Mapourika and Franz X) are from the Franz Josef chronosequence. Both chronosequences are in the South Island, New Zealand.

Figure 1 shows the CP-MASS-¹³C-NMR spectrum of Mapourika soil. ¹³C resonances can be assigned if it is assumed that the isotopic chemical shifts (δ) in such solids are not significantly different (that is ± 2 –3 p.p.m.) from those in solution. The spectrum can be divided into five regions: δ 10–50 p.p.m. (alkyl carbon), δ 50–100 p.p.m. (O-alkyl carbon), δ 100–110 p.p.m. (acetyl carbon), δ 110–160 p.p.m. (alkene and aromatic carbon) and δ 160–200 p.p.m. (carbonyl carbon). The aromatic region consists of two distinct groups of resonances centred around δ 128 p.p.m. and 150 p.p.m. The latter most probably arises largely from aromatic carbons next to oxygen (phenols and furans). The former is protonated carbon plus substituted carbons of aryl carboxylic acids.

The proportions of various carbon types present in the soil determined by integrating the six regions of the spectrum in Fig. 1 are given in Table 2. These values are only approximate estimates for several reasons. First, because resonances from the various carbon types are not fully resolved, it is not possible to integrate the spectra with great accuracy. Second, it is not possible to differentiate between carbons which may be unusually shifted upfield or downfield from their respective common chemical shifts. Third and most important, the cross-polarization process does not necessarily give quantitative data. It must be established that the proton population relaxes with a uniform time constant ($T_{1\rho}$) and that sufficient time delays between repeating pulse sequences allow total relaxation of protons. The rate of cross-polarization depends on the proximity of protons to carbons, hence the contact time between carbons and protons must be optimized to ensure all carbon types are polarized to an equal extent.

Although $T_{1\rho}$ values in soils are short (<10 ms)² there is some evidence that results obtained by CP-MASS-¹³C-NMR are quantitative. In CP experiments maximum enhancement of different carbon types occurs at contact times and recycle times

**Fig. 3** CP-MASS spectra of *a*, Hokitika and *b*, Tirau soil. Spectra were collected in similar conditions as Mapourika soil.

of magnitudes used in the present experiments². In addition, aromaticities of humic substances measured by CP, CP-MASS and conventional solution NMR are similar^{8,9}, and CP-MASS has been widely used in the interpretation of closely related coal samples which have similar relaxation rates^{10–12}.

Table 2 clearly shows that alkyl carbon is an important component of Mapourika soil. Because the alkyl signal is centred around 30 p.p.m. much of this carbon is polymethylene [(CH₂)_n]. The evidence for polymethylene in soils has been reviewed¹³. Aliphatic carbon α to oxygen is also an important component of the soil; about 35% of the carbon is in this form. Small amounts of acetal carbon are also present. The O-alkyl and acetal carbon probably originate from carbohydrates. The ratio of O-alkyl to acetal carbon is 4:1 which, bearing in mind the approximate nature of the results, is consistent with hexose sugar units such as those in cellulose. About 10% of the carbon in Mapourika soil is carboxylic. This is in excellent agreement with typical values obtained for humic acids by conventional functional group analysis¹⁴.

It had been assumed that all carboxylic carbons in soil organic matter are attached to aromatic rings, however, Anderson and Russell¹⁵ suggest that polymaleic acid-like molecules are important components of humic substances; that is, many of the carboxylic groups are attached to alkyl groups. The aromaticity (fraction of carbon which is aromatic, f_a) of the Mapourika soil is low (~15%). The fraction of aromatic carbon is only slightly higher than the fraction of carboxylic carbon (10%). Much of the aromatic carbon is clearly not attached to carboxylic groups, as a significant proportion of the aromatic carbon is oxygenated or protonated. Hence, our results suggest that many of the carboxylic carbons are bound to aliphatic carbon¹⁵.

The spectra of the other soils (Figs 2, 3) show that the structure of soil organic matter can vary widely from soil to soil. The

Table 2 Approximate estimates of carbon types in soils

Soil	Assignment Chemical shift peak	Alkyl 30	O-Alkyl 73	Acetal 103	ArylH* 128	Aryl-R†† 150	f_a —	COOR§ 173	CO 198
Mapourika		0.30	0.35	0.09	0.10	0.05	0.15	0.10	—
Ikamatu		0.26	0.33	0.11	0.11	0.08	0.19	0.08	0.03
Hokitika		0.27	← 0.30 →	→	← 0.37 →	→	0.37	0.06	—
Franz X		0.33	0.33	0.07	← 0.17 →	→	0.17	0.10	—
Tirau		0.30	← 0.37 →	→	← 0.23 →	→	0.23	0.10	—

* Some substituted carbon of aryl carboxylic acids or phenolic aryl carboxylic acids may occur in this region.

† R = O, C.

‡ Largely aromatic oxygenated carbon.

§ R = H, metal ion, alkyl.

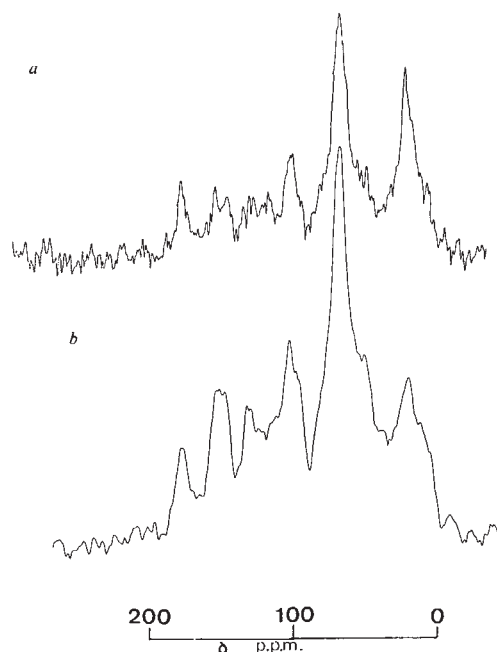


Fig. 4 CP-MASS spectra of soil decomposition products of *a*, beech and *b*, pine leaves (needles). Leaves were aged for 2 yr by microbiological decomposition. Detailed experimental data are available elsewhere¹⁶.

degree to which different functional groups can be resolved depends on the preponderance of one carbon type to another. Most of the detail present in the spectrum of Mapourika soil is also present in the spectrum of Ikamatu soil (Fig. 2*b*) but the greater amounts of acetal and aromatic carbon mean that different carbon types are not so well resolved. The Hokitika soil is a case in point (Fig. 3*a*); about 37% of the carbon is aromatic and 27% alkyl. The *O*-alkyl and acetal carbons are not resolved, nor are substituted and unsubstituted aryl carbon resonances.

Even though unsuitable rotors were used, the three soils (Maungatua, Okarito and Te Kopuru) previously investigated by CP-MASS-NMR³ show enough detail to allow them to be classified with the soils described above. Maungatua humic silt loam is highly aliphatic and similar in organic structure to the Mapourika soil except the *O*-alkyl and acetal carbon content is much lower. The other two soils (Te Kopuru and Okarito) are of more aromatic character and similar to Ikamatu soil. Nevertheless, these soils also show that the *O*-alkyl and acetal content can vary considerably³.

Figures 1 and 2 show that further, qualitative information is available from CP-MASS-¹³C-NMR spectra. Mapourika soil shows evidence of carbon resonances at 61 p.p.m., probably due to $-\text{CH}_2\text{OH}$ carbon. There is also resonance at 55 p.p.m. which is possibly methoxy carbon. The Ikamatu spectrum (Fig. 2*b*) offers very tentative evidence for ketone carbon at δ 198 p.p.m.

Clearly, CP-MASS-¹³C-NMR has enormous potential in soil science. No other analytical technique reveals such structural detail on the nature and forms of organic carbon. More extensive studies than those reported here may clarify the various processes resulting in soil formation. From our results it is too early to comment on genetic relationships between soils, because variations in structure of organic matter between horizons of the same soil may be larger than differences between soils. Nevertheless, we can comment on humification processes. We have investigated the decomposition of plant litter by wet chemical and solid state NMR techniques. Detailed experimental data are available elsewhere¹⁶. The CP-MASS-¹³C-NMR spectra of beech and pine leaves that have been microbiologically aged for 2 yr are shown in Fig. 4. Note that the major carbon types identified in some of the soils described in this study (Figs 1 and 2) are also those present in aged plant litter. The discrete

resonances in Figs 1 and 2 apparently represent decomposing organic matter, and the broad underlying resonances such as those in Fig. 3 represent humified material. Thus our results clearly show that CP-MASS-¹³C-NMR is ideal for studying the degree of humification and humification rates of soils.

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Marshrock formed by iron sulphide and siderite cementation in saltmarsh sediments

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The formation of authigenic sulphides and other iron minerals has proved interesting both in studies of palaeomagnetism¹ and palaeosalinity reconstructions of Pleistocene and older sediments^{2,3}. Iron sulphide and siderite-cemented nodules, concretions and layers are fairly common in the geological record^{4–6}, but there is little evidence of their formation in modern environments. Accounts of authigenic iron minerals forming today in marine and brackish sediments generally refer only to 'micro-concretions' 10–500 μm in size, many of which have a 'framboidal' texture^{3,7,8}. I describe here 'macro-concretions' and cemented layers which are now forming within intertidal marsh sediments at Warham, Norfolk, UK. Parts of these marshes are only 30 yr old, indicating how quickly early diagenetic cementation can occur. Siderite (FeCO_3), greigite (Fe_3S_4) and possibly mackinawite (FeS_{1-x}) are the main cementing agents. The term 'marshrock' is proposed for the cemented sediments by analogy with calcareous beachrock^{9,10}. The formation of siderite-monosulphide concretions in the Warham sediments probably reflects a high 'reactive iron' content and limited sulphate arising from rapid sedimentation and burial of organic matter during the early stages of marsh growth.

The Warham marshes form part of an almost continuous series which extend 35 km between Cley and Holme on the north Norfolk coast. They are open coast marshes, partly protected by barrier islands, spits and intertidal sand flats. Warham has an Upper Marsh (2.7–3.0 m OD) and a Lower Marsh (1.9–2.1 m OD), to seaward of which is an area of unvegetated intertidal sand flats (1.7–1.9 m OD)¹¹. The Upper Marsh and Lower Marsh are covered by ~6% and 55% of tides respectively¹¹.

Cemented deposits have only been observed within the Lower Marsh sediments, and are best exposed along the banks of tidal creeks and a low cliff which marks its seaward edge, especially after spring tides when creek beds are subject to scour. Air photographs show that in 1948 most of the Lower Marsh area was unvegetated and possessed no distinct creek system, but that by 1960 vegetation was well established and the creek system

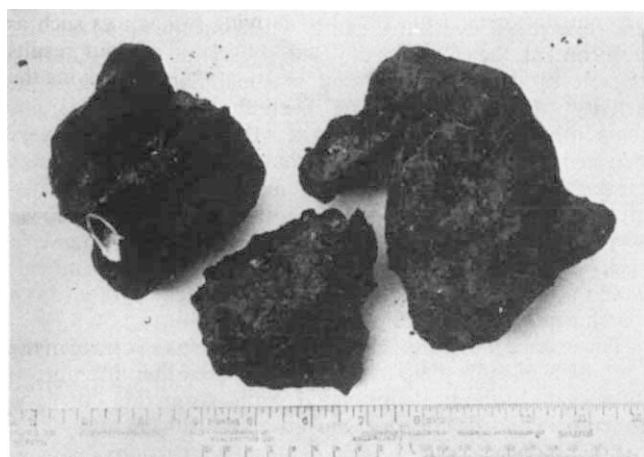


Fig. 1 Iron sulphide and siderite-cemented concretions from the Lower Marsh at Warham. Note the intact shell in the left-hand concretion.

had developed¹². The marsh sediments are therefore mostly <30 yr old and the cemented layers and concretions must have formed within this time.

Observations suggest that cemented sediments occur discontinuously throughout the Lower Marsh area, but not in the Upper Marsh or intertidal flat sediments. The upper 1–1.5 m of Upper Marsh sediments are oxidized, but it is not known whether concretions and layers are present below this zone at elevations corresponding to those on the Lower Marsh. Preliminary observations elsewhere on this coast, however, support the conclusion that active concretion formation takes place only in younger lower marsh sediments.

The uppermost 30–40 cm of the Lower Marsh sediment column at Warham consists of brownish, organic-rich silty sands which overlie at least 1 m of black sands and silty sands. These colour zones correspond respectively to the 'hydroxide' and 'monosulphide' zones previously identified in Dutch coastal sediments¹³ and in the Wash⁹. At Warham no lowermost grey 'bisulphide' zone is visible, but may be present beneath the monosulphide zone at depth.

The concretions and cemented layers are found within the black monosulphide zone, often in its upper part. Concretions are rounded and up to 40 cm in diameter (Fig. 1), but may be larger and more irregular in shape due to intergrowth of two or more neighbouring concretions. In some places continuous concretionary layers 15–25 cm thick can be traced laterally for 3 or 4 m. Many concretions and layers display 3–5 cm long tubular projections which often still contain recognizable rootlets. Some concretions also show clear horizontal grain size laminations which can be traced laterally into adjacent non-cemented sediments. Both these features testify that cementation has occurred within the marsh sediments and that the concretions are *in situ*. Some cemented material has been reworked in areas of localized bank erosion and is found as isolated fragments in creek beds and on the upper part of the intertidal flats. Such concretions usually have extensive surficial oxidation and bryzoan or algal encrustation.

Fresh specimens of cemented material are black though they may contain veins and patches of oxidized brown pigment. The concretions are hard, and there is a fairly sharp transition to the surrounding non-cemented sediment. Many specimens contain intact and broken shells, detrital chert pebbles, and fragments of macro-organic matter.

The material generally has a grain-supported fabric, with medium and fine-sized quartz representing >70% of the framework grains. Scanning electron microscopy (SEM) shows that the framework grains are cemented by rounded, granular aggregates or spherules 2–5 μm in diameter (Fig. 2). The

spherules themselves seem to consist of individual particles up to 0.5 μm in size, many of which have an incomplete cubic form (Fig. 2b). Energy dispersive analysis (EDAX) under the SEM has detected large amounts only of iron and sulphur in these particles, with subsidiary aluminium, silicon and manganese.

The cemented sediments disintegrate completely within a few hours, with emission of H_2S and CO_2 , when allowed to stand in cold HCl, suggesting that pyrite and marcasite, which are insoluble in HCl ^{2,14}, are not major components of the cement. Greigite, mackinawite and siderite are HCl-soluble, however. X-ray diffraction data (Table 1) confirm the presence of siderite and greigite, but do not confirm mackinawite or other form of FeS. The presence of a monosulphide other than greigite is suggested indirectly by the fact that some of the cement oxidizes rapidly to a brown colour on exposure to air, whereas greigite is stable in air and can withstand heating to 238 °C for 165 h without alteration¹⁵. Several unidentified X-ray peaks, which disappear after HCl treatment, may represent mackinawite or other monosulphide, although they do not correspond exactly to previous data for such minerals.

Siderite is apparently the major constituent of the cement in many concretions, although greigite and monosulphides are probably responsible for the black coloration. Approximately 20% of a sample of freshly crushed cement was attracted by a magnet, a property characteristic of greigite but not of siderite or mackinawite¹⁵.

Both the formation of concretions and the dominance of siderite and greigite rather than pyrite in the Warham Lower Marsh may be explained in terms of the balance between 'reactive' iron and available sulphate (and therefore H_2S) within the sediments. Berner¹⁶ has demonstrated that both concretionary and layer concentrations of iron sulphide will probably

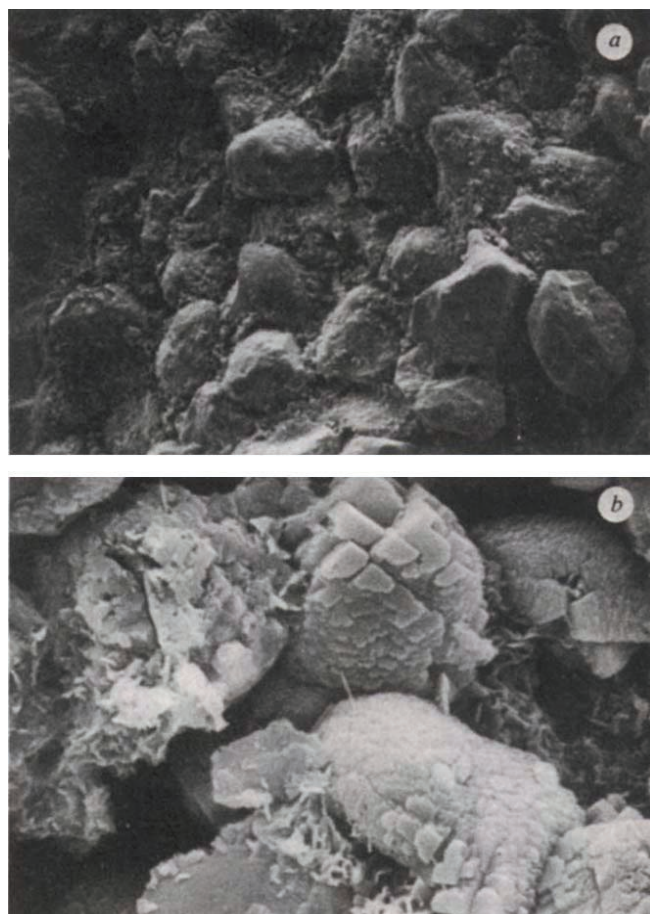


Fig. 2 Scanning electron micrographs showing: a, quartz framework grains and iron sulphide-siderite cement (picture width 1.3 mm); b, the cubic nature of some individual constituent particles of the iron sulphide spherules (picture width 100 μm).

Table 1 XRD data for two samples of marshrock cement (CuK α radiation)

a dÅ	b dÅ		
5.52 s	5.42 vs		
5.37 s	5.32 s		Greigite?
3.82 vw	3.79 vw		
3.66 s	3.64 s	Siderite	
3.62 s	3.60 s		Greigite
3.22 vw	3.19 s		
3.02 s	2.99 m		Greigite
2.89 vw	2.85 w		
2.82 vvs	2.81 vvs	Siderite	
2.46 m	2.46 vw		Greigite
2.36 s	2.36 w	Siderite	
	2.25 w		
2.15 s	2.14 w	Siderite	
	2.09 vw		
1.98 s	1.97 w	Siderite	
1.86 w			Greigite
1.81 w	1.80 vw	Siderite	
1.75 s	1.74 m	Siderite	Greigite
	1.67 vvw		
	1.59 vvw		
1.52 s	1.51 vw	Siderite	Greigite

vs, Very strong; s, strong; m, moderate; w, weak; vw, very weak.

form where there is a relatively high ratio of reactive iron to reducible sulphur. In such conditions outward diffusion of H₂S from an organic-rich source area is restricted by immediate reaction with dissolved Fe²⁺ and resultant precipitation of FeS close to the source area. Because FeS is relatively insoluble, the concentration of dissolved iron in this area is reduced, thereby creating a diffusion gradient which allows further iron to move towards the incipient concretion.

The first-formed iron sulphide precipitates, mackinawite and greigite, are thermodynamically unstable relative to pyrite and should be converted to the latter by reaction with polysulphide ions¹⁷⁻¹⁹. However, this diagenetic transformation can be arrested where sulphate availability is limited and monosulphides may be preserved²⁰. This situation may occur in seawater but is most common in freshwater lakes and estuaries which contain less dissolved sulphate. Moreover, sediments which possess a high concentration of dissolved Fe²⁺, and in which the supply of H₂S rapidly becomes depleted, may attain supersaturation with regard to non-sulphide iron minerals such as siderite and vivianite (Fe₃(PO₄)₂·8H₂O), thereby leading to their precipitation²¹⁻²⁴.

In saltmarshes which do not occur within brackish water estuarine settings, as at Warham, the most important factor leading to rapid depletion of sulphate and H₂S is probably a high rate of sediment accretion. For any given sulphate concentration, more rapid accretion results in more rapid bacterial sulphate reduction due to burial of more highly reactive organic matter^{25,26}. If deposition is sufficiently rapid, the rate of sulphate reduction may be so great that the sulphate supply is exhausted before FeS is completely altered to pyrite^{1,26}. On the north Norfolk coast, accretion rates on marshes less than 30 yr old are of the order of 1.0–1.7 cm yr⁻¹ (ref. 27), and may provide the conditions necessary for rapid sulphate exhaustion, monosulphide preservation and siderite formation. On older and higher marshes, accretion rates are much lower, of the order of 0.002 cm yr⁻¹ (ref. 27), and suitable conditions for these diagenetic processes are much less likely. This would explain why no concretions appear to be actively forming within the Upper Marsh at Warham and other 'high' marshes on the Norfolk coast. Because marsh accretion rates are initially most rapid and seem to decrease exponentially with time²⁷, siderite/greigite concretion formation may only occur in sediments deposited during the first few decades of marsh growth.

The nature of authigenic iron minerals formed in salt marsh sediments will depend on several factors other than accretion

rate (which reflects sediment availability and type, tidal regime and relative sea level movements), which include initial reactive iron content, the character of marsh vegetation (which controls organic production rates), and seasonal fluctuations in the activity of sulphate-reducing bacteria (which are largely temperature-controlled). Because these conditions vary between regions, marshes in different areas of similar age may possess distinctive suites of authigenic iron minerals. Siderite-greigite concretions and layers are unlikely to form in slowly accreting marsh sediments with a low reactive iron content; in such situations disseminated pyrite crystals and framboids would probably form very quickly, as has been reported from marsh peats in the eastern US²⁸.

The apparent importance of accretion rate as a control in the formation of authigenic iron minerals means that attempts to infer palaeosalinities associated with formation of iron concretions in fossil deposits must proceed with caution.

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Ipswichian fauna of Victoria Cave and the marine palaeoclimatic record

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The lack of age dating methods which can be applied beyond the limit of radiocarbon dating makes the global correlation of continental climatic events and stratigraphic sequences with the continuous palaeoclimate record, obtained by isotopic and faunal analysis of deep-sea sediment cores¹⁻³, a difficult task; often only a relative time scale can be obtained using complex and perhaps tenuous litho- and biostratigraphical data. Speleothems may assist in this correlation, for embodied in their calcium carbonate structures are elemental and isotopic variations which indicate both their age and climatic conditions prevailing during their deposition. Speleothems may be reliably dated by the ²³⁰Th/²³⁴U method to an age of ~350 kyr BP providing they consist of non-porous, unrecrystallized calcite which is essentially free of clastic detrital sediments⁴⁻⁶. They

may also provide palaeoclimatic information for previously glaciated areas (1) because their presence indicates that groundwater movement was not prevented by ice formation at the surface, and CO₂ was produced in the soil zone by root respiration and plant decay (limestone bedrock may then be dissolved and reprecipitated as speleothems in caves below the surface), and (2) because of the variations in stable isotopic content and fluid inclusion waters contained therein^{4,7,8}. Additional climatic and chronological information may be obtained where speleothems are interstratified with deposits characteristic of a particular climatic regime, and with deposits containing organic remains. We describe here the results of dating speleothems encrusting the remains of mammals from Victoria Cave in northern England, and discuss their significance in terms of the correlation between continental and oceanic palaeoclimatic records.

Victoria Cave is situated at an altitude of 440 m a.s.l. in a Carboniferous limestone scar north-east of Settle, North Yorkshire. The Victoria Cave Exploration Committee of the British Association for the Advancement of Science conducted excavations at the site from 1870 to 1878 and published details of the Pleistocene deposits⁹. Two quite distinct fossiliferous horizons were found, but we will consider here only the older of these, the Lower Cave Earth. Tiddeman⁹ records an interglacial mammal fauna from this deposit which included such animals as hyaena, hippopotamus and narrow-nosed rhinoceros. He observed that the Lower Cave Earth was truncated at the cave mouth by glacial till, while inside the cave it was overlain by laminated clays. It was reasoned that the till and laminated clays were laid down during the last glacial period and that the lower Cave Earth and its contained fauna belonged to the preceding interglacial. The relationship of these deposits is shown in Fig. 1.

Further (unpublished) excavations by Lord in 1937 revealed mammal bones, teeth and deer antler lying on a surface within the Lower Cave Earth, covered by a layer of calcite flowstone to a depth of ~10 cm. Their unweathered condition indicated that the time between their introduction into the cave and their burial beneath the flowstone was not great. All the samples from which the present series of ²³⁰Th/²³⁴U dates are derived were taken from blocks found during Lord's excavations, now housed in the Pigyard Museum at Settle. Re-examination of material from Victoria Cave has confirmed the presence of the above mammals recorded by Tiddeman in the Lower Cave Earth (and its interbedded speleothems). A list of the Victoria fauna is given in Table 1.

This faunal assemblage is a classic example of the late Pleistocene 'hippopotamus fauna' as defined by Sutcliffe¹⁰ on the basis of material from Joint Mitnor Cave in Devon. He assigned the assemblage to the warmest part of the Eemian (= Ipswichian) interglacial. Palaeobotanical evidence from Trafalgar Square, London¹¹ and Barrington, Cambridgeshire¹² has demonstrated the association of the 'hippopotamus fauna' with

Table 1 Fauna of the Lower Cave Earth in Victoria Cave

Species identified	Common name
<i>Ursus arctos</i>	Brown bear
<i>Crocuta crocuta</i>	Spotted hyaena
<i>Panthera leo</i>	Lion
<i>Palaeoloxodon antiquus</i>	Straight-tusked elephant
<i>Dicerorhinus hemitoechus</i> *	Narrow-nosed rhinoceros
<i>Hippopotamus amphibius</i> *	Hippopotamus
<i>Megaloceros giganteus</i> *	Giant deer
<i>Cervus elaphus</i> *	Red deer
<i>Bos</i> or <i>Bison</i> sp.	A bovine

* Species present in the dated flowstone samples.

interglacial pollen spectra representing a period of climatic optimum (zone Ip IIb). At Swanton Morley, Norfolk, there is evidence for the continued existence of *Hippopotamus* into the declining phase of the interglacial (zone Ip III)¹³.

The 'hippopotamus fauna', which is represented at many localities in England and Wales, is remarkably constant in its composition and there is no evidence to suggest that this particular species grouping was present more than once in the British sequence. Many fluviatile deposits in southern Britain have produced an extraordinary abundance of remains of hippopotamus, yet it is virtually absent from similar deposits in adjacent parts of the continent strongly suggesting that we are dealing with an island fauna, and therefore, a period in which sea-level was sufficiently high to isolate Britain from Europe. Rare occurrences of *Hippopotamus* in France, in apparent 'Riss-Würm' contexts, may chart this animal's route from the Mediterranean area.

At Sewerby Cliff, Holderness, elements of the 'hippopotamus fauna' have been found in beach deposits in front of a buried cliff feature at 2–3 m above OD, slightly higher than the level of modern high water during spring tides at the same locality¹⁴. The elevation of the deposits suggests a correlation with the high sea-stand of isotope substage 5e¹, thus precluding a younger age for the 'hippopotamus fauna'. However, their elevation may be due, in part, to local isostatic movement and crustal adjustment, so that correlation with the younger substages, 5c and 5a, cannot be completely ruled out unless the deposits can be assigned an absolute age. This has been possible in the case of the Victoria Cave fauna by dating the speleothems associated with the bone remains.

Seven samples of flowstone were analysed by the ²³⁰Th/²³⁴U dating method, using analytical techniques and age calculations described elsewhere¹⁵. The accuracy and precision of ages determined by this method are demonstrated by: (1) 12 analyses of a homogenized speleothem standard (mean age 47.8 kyr BP) have shown that the mean error determined by averaging 1σ counting uncertainties (±2.2 kyr) is comparable to the standard deviation of the determined ages (±1.7 kyr)¹⁶; (2) close agreement of ages determined by the McMaster laboratory with other laboratories for several homogenized carbonate standards has been found in the uranium-series interlaboratory comparison project¹⁷, and (3) results of duplicated analyses of the same sample of several U-rich speleothems using the ²³¹Pa/²³⁰Th dating method show good agreement with ²³⁰Th/²³⁴U ages¹⁶.

All samples analysed consist of yellow to brown laminated calcite enclosing or overlying bones. Sections cut through the flowstones clearly showed that the calcite post-dated the bone. The results of 12 analyses are shown in Table 2. Except for three results (79000-1, 79001-1 and 79025-1) all ages fall within the interval 135–114 kyr BP. Re-determination of these three apparently younger samples gave ages within the 135–114 kyr interval (79000-2, 79001-3, -4, 79025-3). The initial younger ages are thought to be due to slight contamination by uranium which had migrated from the relatively U-rich fossil bones into the immediately adjacent calcite. The replicates were taken from calcite more removed from (and therefore stratigraphically younger than) the initial analyses, and hence were less likely to

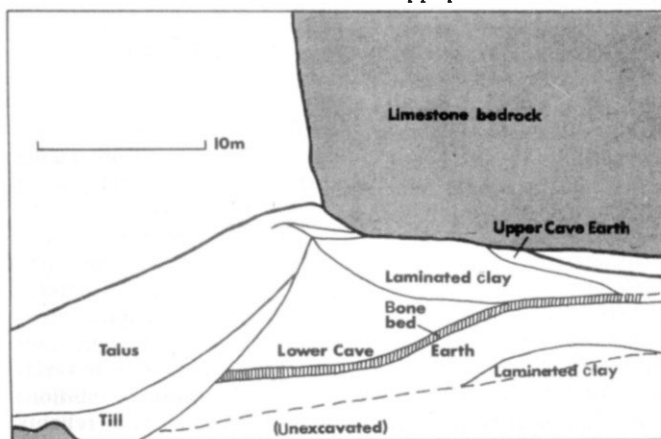


Fig. 1 Diagrammatic longitudinal section of the Pleistocene deposits of Victoria Cave.

Table 2 Sample descriptions, isotopic ratios and $^{230}\text{Th}/^{234}\text{U}$ ages for seven samples of flowstone containing mammal remains from Victoria Cave, northern England

Speleothem no.	Description	No.	Analysis Location	U (p.p.m.)	$^{234}\text{U}/^{238}\text{U}$	$^{230}\text{Th}/^{232}\text{Th}$	$^{230}\text{Th}/^{234}\text{U}$	Age (kyr)
79000	Brown laminated flowstone enclosing teeth of <i>Dicerorhinus hemitoechus</i>	-1	Adjacent to teeth	0.62	1.000 ± 0.067	44	0.610 ± 0.041	102^{+12}_{-11}
		-2	~1 cm away from teeth	0.63	1.019 ± 0.021	20	0.672 ± 0.021	120 ± 7
79001	Yellow-brown flowstone enclosing jaw of <i>Dicerorhinus hemitoechus</i>	-1	Adjacent to bone	0.50	1.100 ± 0.028	20	0.623 ± 0.022	104 ± 6
		-2	~2 cm away from bone	0.40	1.033 ± 0.031	152	0.691 ± 0.027	126^{+10}_{-9}
		-3	~2 cm away from bone	0.43	1.022 ± 0.029	34	0.714 ± 0.022	135 ± 8
79002	Calcite enclosing antler of <i>Cervus elaphus</i>	-1	Adjacent to bone	0.50	1.012 ± 0.026	16	0.702 ± 0.024	131^{+9}_{-8}
79021	Flowstone enclosing teeth of <i>Dicerorhinus hemitoechus</i>	-1	2 cm adjacent to teeth	0.43	1.057 ± 0.024	31	0.690 ± 0.019	125^{+7}_{-6}
79023	Flowstone and pool calcite enclosing teeth of <i>Megaloceros</i>	-1	1 cm of clean side of flowstone	0.50	0.994 ± 0.022	71	0.678 ± 0.020	123 ± 7
79025	9-cm deep section of flowstone block which contains large mammal bones at its base including calcaneum of <i>Hippopotamus</i>	-1	0.5 cm above base (at bone level)	0.88	1.120 ± 0.021	111	0.484 ± 0.014	71 ± 3
		-2	Top 1.5 cm	0.43	1.048 ± 0.019	84	0.654 ± 0.016	114 ± 5
		-3	0.5 to 1.5 cm above base	0.58	1.037 ± 0.018	47	0.672 ± 0.019	120 ± 6
79026	Flowstone enclosing teeth of <i>Dicerorhinus hemitoechus</i>	-1	Top (?) 2 cm of flowstone	0.46	1.022 ± 0.016	>1,000	0.668 ± 0.016	119 ± 5

* U yield = 6%. All other yields are >20%. All error limits are $\pm 1\sigma$.

be influenced by nuclide migration from the bone. The appreciably higher U content of the most anomalous age determination (79025-1) further supports this interpretation, although this distinction is not seen in the case of 79000.

The results indicate that members of the 'hippopotamus fauna' were present in the vicinity of Victoria Cave during or before the interval 135 ± 8 to 114 ± 5 kyr BP. More specifically, because the basal and top ages of sample 79025 are in normal stratigraphic sequence, and because the bone layer lies at the base of this deposit, the fauna of the Lower Cave Earth can be placed at or before 120 ± 6 kyr BP. This compares very favourably with the date of $125\text{--}120$ kyr BP for substage 5e of the marine isotopic record^{1,18}.

The association of Ipswichian pollen assemblages with the 'hippopotamus fauna' at several sites would suggest that the Ipswichian interglacial *sensu stricto* must therefore also coincide with substage 5e. However, the paucity of vertebrate fauna at the Ipswichian type locality at Bobbitshole, near Ipswich, Suffolk, makes a direct correlation with the Lower Cave Earth in Victoria Cave impossible. Elsewhere, there are reported discrepancies between what are apparently Ipswichian pollen assemblages and the associated mammal faunas, suggesting that some of the pollen assemblages assigned to the Ipswichian may actually represent other warmer stages^{19,20}. Many of these difficulties arise from the unsuitability of the chosen type locality for this important phase of the Pleistocene. Despite such problems, the term 'Ipswichian' is widely applied to the last period in which the climate of the British Isles was as warm as, or warmer than, at present, though this is not strictly within the original definition of the term. This procedure is necessitated by the absence of any other acceptable name for the same phase. With this qualification, we feel justified in correlating the 'Ipswichian interglacial' of the British terrestrial sequence with substage 5e of the marine isotope record.

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Phosphotyrosine-containing proteins isolated by affinity chromatography with antibodies to a synthetic hapten

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Tyrosine-specific protein kinases seem to be involved critically in cellular transformation by tumour viruses^{1–4}, and may also be involved in the cellular response to epidermal growth factor⁵. To facilitate the identification and isolation of phosphotyrosine-containing proteins (PT-proteins) we have sought to develop as a general reagent anti-O-phosphotyrosine antibodies. We show here that affinity chromatography with these antibodies is effective for the small-scale isolation of some PT-proteins, including p120, the transforming protein of Abelson murine leukaemia virus, and several unidentified proteins from Rous sarcoma virus (RSV)-transformed mouse fibroblasts.

New Zealand rabbits were injected repeatedly intradermally (i.d.), each time with ~1 mg of keyhole limpet haemocyanin containing ~30 *p*-azobenzyl phosphonate⁶ (ABP) groups per 100,000 molecular weight (*M_r*). Benzyl phosphonate, rather than phenyl phosphate, was chosen to avoid the loss of haptenic groups by the action of tissue phosphatases. For the first injection, the antigen was incorporated in complete Freund's adjuvant; for subsequent ones (on days 23, 33, 40 and 92) it was given in incomplete Freund's adjuvant. Anti-ABP activity in serum was monitored by a solid phase radioimmunoassay in which small tubes (Dynatech) coated with ABP₃₀-bovine serum

albumin (BSA) were incubated with the rabbit antisera, washed and finally tested with ^{125}I -labelled *Staphylococcus aureus* Protein A to measure the extent of binding of rabbit immunoglobulin to the ABP-BSA.

Antibodies were isolated from the active sera by affinity chromatography on Sepharose to which was attached *O*-phosphotyramine. The latter was prepared by phosphorylating tyramine with P_2O_5 (ref. 7). A strongly coloured side-product was precipitated by the addition of 1 vol ethanol and 13 vol *n*-butanol. *O*-phosphotyramine was then precipitated by addition of 15 vol ethyl acetate and recrystallized twice from ethanol/water. The recrystallized product migrated as a single component on TLC using isopropanol/ H_2O /conc. NH_4OH (7:2:1) as solvent. On silica gel ($R_F = 0.1$) it was detected using I_2 , ninhydrin and by UV absorption, and on cellulose ($R_F = 0.2$) it was detected with ninhydrin. Its NMR spectrum was consistent with the expected structure. The recrystallized *O*-phosphotyramine was coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (30 mg per g) in 0.1 M NaHCO_3 . Immunoglobulins from anti-ABP serum (precipitated with 40%

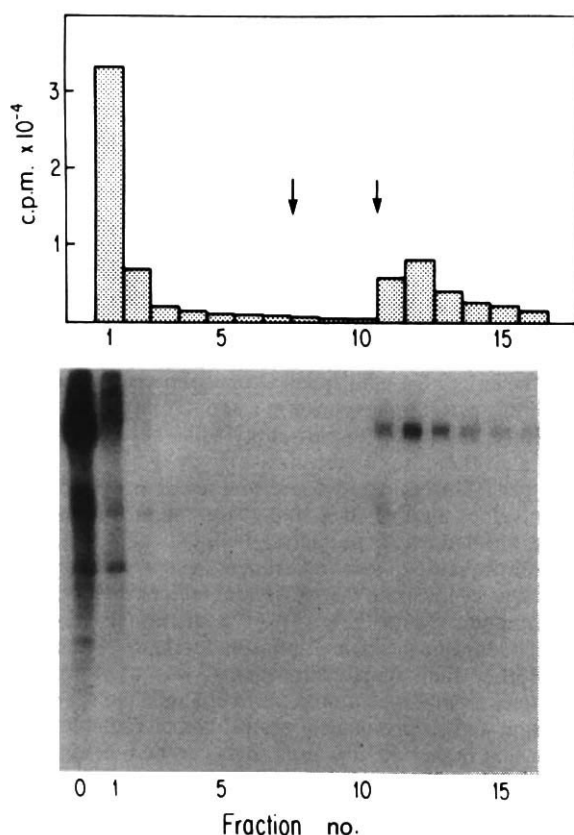


Fig. 1 Affinity chromatography of phosphotyrosine-containing proteins from an extract of Abelson virus-transformed B lymphocytes (ANN-1 cells). A crude preparation of p120 (40 μg) was phosphorylated by reaction for 30 min on ice with 5 μCi [γ - ^{32}P]ATP in 10 mM MnCl_2 , 1 mM phenylmethylsulphonyl fluoride (PMSF), 100 kallikrein units aprotinin ml^{-1} , 150 mM Tris pH 8.0. After addition of 20 mM $\text{Na}_2\text{-EDTA}$, the reaction mixture was diluted with an equal volume of buffer I (0.5% NP-40, 150 mM NaCl, 100 units aprotinin ml^{-1} , 0.1 mg ml^{-1} ovalbumin, 10 mM Tris pH 8.0) and applied to a 0.15 ml Sepharose column of anti-ABP antibodies (0.75 mg immunoglobulin per ml Sepharose). The column was washed with buffer I (fractions 2–7), then with buffer II (fractions 8–10) and finally with buffer III (fractions 11–16). Buffer II differed from I only in that II contained 20 mM phosphoserine, 20 mM phosphothreonine, 50 mM NaCl and 3.3 mM Na_2HPO_4 . Buffer III differed from II in that III had 40 mM phenyl phosphate in place of phosphoserine and phosphothreonine. Each fraction was 0.5 ml. The protein precipitated with 5% perchloric acid from a 25 μl sample of each fraction was collected on a Millipore filter and counted in Bray's solution (upper panel). Of the total perchloric acid-precipitable radioactivity applied to the column, ~50% was recovered. A sample of each fraction and of the original reaction mixture ('fraction 0') was also analysed by electrophoresis in 10% SDS-polyacrylamide gels¹³, followed by autoradiography of the dried gels (lower panel). The 'fraction 0' sample had half the volume of the other fractions.

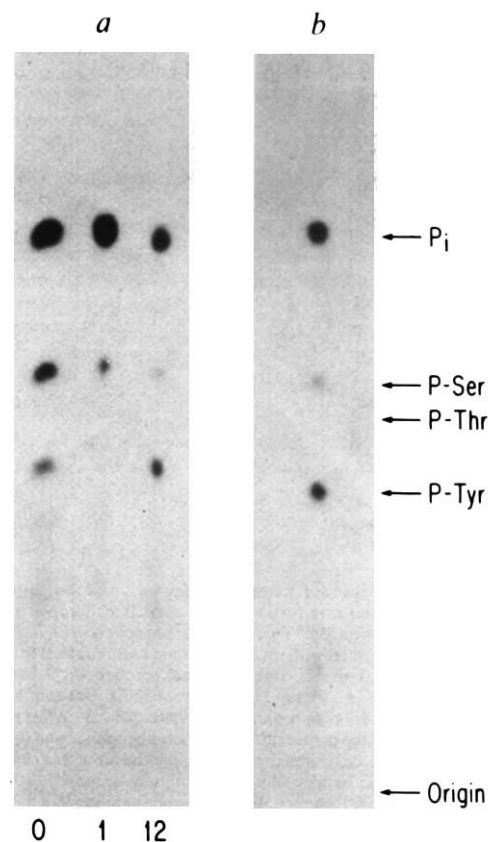


Fig. 2 Phosphoamino acid analyses by high voltage paper electrophoresis. Proteins were precipitated with 5% perchloric acid and washed successively with 5% perchloric acid, 5% trichloroacetic acid and acetone. The samples were hydrolysed for 2 h at 100 $^{\circ}\text{C}$ in 6M HCl, dried and subjected to high voltage electrophoresis in pyridine-acetic acid (pH 3.5) on Whatman 3-MM paper at 2,400 V for 1 h. *a*, Samples from the reaction mixture (fraction 0), and fractions 1 and 12 of the column described in Fig. 1. *b*, Phenyl phosphate-eluted material from RSV-transformed fibroblasts labelled as intact cells with ^{32}P -inorganic phosphate (see Fig. 3 legend). P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

saturated ammonium sulphate) were applied to the phosphotyramine-Sepharose column at room temperature. After washing the column with buffer (150 mM NaCl, 10 mM NaH_2PO_4 pH 7.4) at room temperature and then at 45 $^{\circ}\text{C}$, the retained antibodies were eluted at 45 $^{\circ}\text{C}$ with modified buffer containing 40 mM phenyl phosphate, 50 mM NaCl and 3.3 mM NaH_2PO_4 pH 7.4. Phenyl phosphate was removed from the eluate by dialysis. The hapten-eluted protein accounted for

Table 1 Specificity of anti-ABP antibodies measured by competition radioimmunoassay

Competitor	% Inhibition
Phosphotyrosine (25 μM)	2
Phosphotyrosine (74 μM)	8
Phosphotyrosine (220 μM)	16
Phosphotyrosine (670 μM)	28
Phosphotyrosine (2 mM)	48
Phosphoserine (2 mM)	<1
Phosphothreonine (2 mM)	<1

Anti-ABP antibodies (3.3 μg in 50 μl phosphate-buffered saline (PBS)) were incubated in polystyrene tubes (Dynatech) for 1 h at room temperature. After washing, the tubes were incubated for 1 h at room temperature with 9 ng ^{125}I -(azophenyl phosphate)- γ -RNase and the above inhibitors in 20 μl PBS. The tubes were then washed and counted. In the absence of inhibitors ~30% of ^{125}I -labelled probe was bound. The high concentration of phosphotyrosine required for inhibition was probably due to the multivalency of the azophenyl phosphate-RNase.

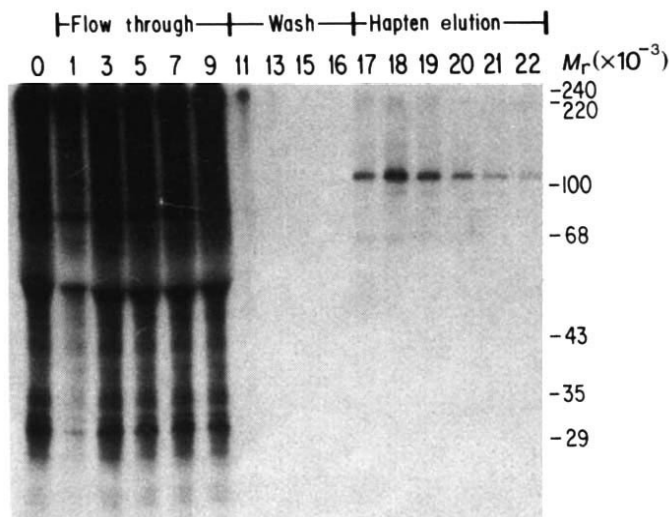


Fig. 3 Isolation of phosphotyrosine-containing proteins from RSV (Schmidt-Ruppin strain)-transformed mouse (BALB/c) fibroblasts; 3×10^6 cells were incubated with 0.25 mCi ^{32}P in phosphate-free medium for $3\frac{1}{2}$ h. The cells were washed with ice-cold medium and extracted three times with 1 ml ice-cold 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.1% BSA, 10 mM $\text{Na}_2\text{-EDTA}$, 10 mM NaF, 150 mM NaCl, 100 units aprotinin ml^{-1} , 1 mM PMSF, 10 mM sodium phosphate pH 7.3. After scraping residual material from the plastic surface with a rubber policeman the extract was homogenized (twelve strokes in a Dounce homogenizer) and centrifuged at 105,000g for 1 h. The supernatant was generally stored at -20°C after addition of glycerol to 30% (v/v). After thawing, samples were mixed with 1 vol buffer I (see Fig. 1) and applied to the column (fractions 1–5), followed by additional buffer I (fractions 10–16) and finally by elution with buffer III (fractions 17–22), which contained phenyl phosphate. The protein from 90 μl of each 0.5 ml fraction was precipitated with 1 ml acetone (-20°C), boiled for 2 min in SDS-2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis. This figure shows the autoradiograph of the dried gel. Sample 0 is the 105,000g supernatant of the total cell extract. Molecular weight markers are indicated at the right. The faint band at $M_r = 36,000$ might correspond to the ^{32}P -p36 protein described elsewhere^{14,15}.

~95% of the ABP-binding activity of the immunoglobulin fraction and corresponded to 0.9 mg antibody per ml serum. By fluorescence quenching⁸ the affinity (equilibrium association constant) of the purified antibodies for the 2,4-dinitrophenyl (DNP) derivatives⁹ of *p*-aminobenzyl phosphonate and of *p*-aminophenyl phosphate was estimated to be $\sim 4 \times 10^6 \text{ mol}^{-1}$ at 20°C . (Because of the simplicity of these ligands and the DNP moiety which was introduced to facilitate affinity measurement¹⁰, the observed equilibrium constants are only approximations of the affinity for phosphotyrosine in PT-proteins.) These ligands did not specifically quench the fluorescence of anti-phenyl arsenate antibodies. As judged by competitive radioimmunoassay with ^{125}I -(azophenyl phosphate)- γ -ribonuclease as the radiolabelled ligand, the anti-ABP antibodies had no measurable affinity for *O*-phosphoserine or *O*-phosphothreonine (Table 1). (The azophenyl phosphate-ribonuclease was prepared from diazotized *p*-aminophenyl phosphate, which was synthesized as described above for *O*-phosphotyramine.)

An 'affinity' column, prepared by coupling the purified anti-ABP antibody to CNBr-activated Sepharose, was tested for its ability to bind a crude preparation of the transforming protein (p120) of Abelson murine leukaemia virus. This protein (given by A. Dasgupta) was phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in conditions where essentially all the ^{32}P incorporated into p120 was in the form of *O*-phosphotyrosine². The ^{32}P -p120 was retained by the column, from which it was specifically eluted by phenyl phosphate, but not by phosphoserine or phosphothreonine (Fig. 1). In other experiments (not shown), neither 40 mM *p*-nitrobenzoic acid nor 10 mM sodium phosphate eluted the p120. Moreover, phosphitin, which is rich in phosphoserine and phos-

phothreonine but lacks *O*-phosphotyrosine, did not bind to the affinity column. The failure of the phosphorylated p120 to adhere to a similar Sepharose column that had been prepared with anti-phenyl arsenate antibodies in place of the anti-benzyl phosphonate antibodies provided additional evidence of specificity. (Antibodies to phenyl arsenate have been reported to cross-react strongly with phenyl phosphonate¹¹; because these antibodies did not retain PT-proteins they probably react only weakly with benzyl phosphonate and phenyl phosphate.)

Aliquots of the column fractions described in Fig. 1 were hydrolysed with 6 M HCl and analysed by high voltage paper electrophoresis (Fig. 2a). *O*-phosphotyrosine was virtually absent from hydrolysates of the pass-through from the affinity column and was enriched in the ^{32}P -protein eluted by phenyl phosphate.

The affinity column was also able to bind ^{32}P -PT-proteins that had been formed by incubating intact cells with ^{32}P -labelled inorganic phosphate (P_i). Schmidt-Ruppin (RSV)-transformed mouse fibroblasts labelled in this way, were lysed by detergent and the lysates applied to the affinity column. Figure 3 shows an SDS-polyacrylamide gel electrophoretic analysis of the proteins that were applied to and eluted from the column, and Fig. 2b shows the ^{32}P -phosphoamino acids in hydrolysates of the eluted proteins. It is evident that the affinity column selected a discrete set of phosphoproteins highly enriched in *O*-phosphotyrosine. One of the conspicuous PT-proteins detected (PT-110) has not to our knowledge been previously reported, perhaps because of its high molecular weight ($M_r = 110,000$; Fig. 3)¹². Because the amount of PT-110 isolated from the RSV-transformed cells was much greater than that obtained from uninfected cells (Y. Onodera, personal communication), PT-110 may be a substrate for pp60^{src}, or for another kinase activated by pp60^{src}, and may play a part in expression of the transformed phenotype.

The specific retention of PT-proteins by antibodies to benzyl phosphonate depends on cross-reactions in which the antibodies have sufficiently high affinity for *O*-phosphotyrosine and negligible affinity for *O*-phosphoserine and *O*-phosphothreonine. The intrinsic affinity for *O*-phosphotyrosine has to be relatively high because there is probably only a single *O*-phosphotyrosine residue per PT-protein and, therefore, few if any opportunities for multivalent binding. It is likely that the anti-benzyl phosphonate antibodies do not have the same affinity for every PT-protein because of local differences in charge density and in accessibility of phosphotyrosine residues. Nonetheless, it seems from preliminary data that ~50–60% of the ^{32}P -PT-proteins were recovered in the phenyl phosphate eluate. Phenyl phosphate, rather than denaturing agents, was used to recover PT-proteins from the column so as to enhance the specificity of the elution and to prolong the useful life of the column. It is possible that higher affinity antibodies can be generated using haptenic groups, for example, *p*-azophenyl phosphate, that are closer in structure to phosphotyrosine than *p*-azobenzyl phosphonate.

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Hot spots of frameshift mutations induced by the ultimate carcinogen *N*-acetoxy-*N*-2-acetylaminofluorene

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An important step in carcinogenesis is thought to be the initial attack of the DNA molecule by a so-called ultimate carcinogen. More than 90% of the carcinogens tested have been found to be mutagens in bacterial systems¹. The covalent binding of the ultimate carcinogen to the DNA bases or phosphate groups creates a premutational lesion that *in vivo* is processed by the repair, replication and recombination enzymes, and eventually may be converted into a mutation. Being interested in the way that an initial premutational event is converted into a stable heritable mutation, we have sequenced stable mutations in a gene that has formed covalent adducts *in vitro* with *N*-acetoxy-*N*-2-acetylaminofluorene (*N*-AcO-AAF, a model for the ultimate metabolite of the rat liver carcinogen 2-acetylaminofluorene AAF). We show here that the mutations are mainly frameshifts involving G·C base pairs, and that certain base pairs (hotspots) are affected at relatively high frequencies. These results fit a model in which *N*-AcO-AAF-modified guanine acts as a non-coding base that during replication results in deletion of the modified residue.

In vivo studies have shown the mutagenicity of AAF and its derivatives in both bacterial^{2,3} and eukaryotic⁴ systems. *N*-AcO-AAF reacts *in vitro* with DNA leading mainly to the formation of a guanine adduct⁵, *N*-2-(deoxyguanosin-8-yl)-acetylaminofluorene (80%) and to at least three minor adducts (N.S., R.P.P.F. and M.P.D., unpublished results), one of which has been characterized as 3-(deoxyguanosin-*N*²-yl)-acetylaminofluorene⁶. Studies by our group showed that binding of *N*-AcO-AAF to DNA resulted in a local distortion of the DNA helix around the C-8 adduct⁷⁻⁹, which we have called the insertion-denaturation model¹⁰. A similar model has been proposed by other investigators¹¹. We describe here the analysis

of forward mutations induced in the tetracycline-resistance gene of pBR322 by directing the chemical reaction of the carcinogen to a small restriction fragment (*Bam*HI-*Sal*I) inside the antibiotic-resistance gene. These two restriction enzymes each cut pBR322 only once and both sites reside in the tetracycline-resistance gene. The strategy used is outlined in Fig. 1. The resulting small restriction fragment (275 base pairs (bp); 6S fragment) modified to various extents with *N*-AcO-AAF (³H ring) was reinserted by *in vitro* ligation into the non-reacted large (*Bam*HI-*Sal*I) pBR322 restriction fragment (16S fragment). The ligation mixture was used to transform CaCl₂-treated *Escherichia coli* recipient cells. Mutants are selected for ampicillin (Ap) resistance and tetracycline (Tc) sensitivity. The induction of SOS functions by UV irradiation of the cells before transformation increased the observed mutation frequency. The plasmid DNA of such mutants was analysed for sequence changes in the fragment where the AAF binding had been directed.

Mutation frequencies were calculated as the ratio of Ap^R Tc^S/Ap^R clones. The frequency in the control experiment in which the 16S fragment was ligated to a non-modified 6S fragment was 0.4% when the bacteria were not treated with UV before the transformation step; when UV treatment was applied, the frequency was 0.6%. When analysed by gel electrophoresis, the plasmid DNAs isolated from such clones were always shorter than the original pBR322, in general by 0.2–0.8 kilobases (kb). The restriction analysis pattern showed that these mutant DNAs had retained the unique *Eco*RI site but that in general they had lost both the *Bam*HI and *Sal*I restriction sites. We call these mutants class I mutants and suggest that they mainly arise from the dimerization of the 16S fragment. Such dimers, which have the Ap^R Tc^S phenotype, are then converted to smaller plasmids (monomers) through *in vivo* recombination. Work is in progress to lower this mutation background by using an alkaline phosphatase-treated 16S fragment¹². Class I mutants were easily recognized and excluded from the pool of mutants to be sequenced.

When 6S-AAF fragments ligated to the non-modified 16S fragment are used to transform *E. coli* the transformation efficiency decreases with increasing levels of bound AAF residues (Fig. 2). The extent of this AAF-dependent inactivation of transformation is strongly related to the general repair genotype of the recipient cell (R.P.P.F. and E. Seeberg, in preparation). One also finds a corresponding increase in the mutation

Table 1 Properties of various mutants

Description of mutation	Mutant no.	Strain	Induction of SOS functions	Average no. of bound residues per 6S fragment	Sequence near the mutation
-1 deletion of G 520 or 521	4	AB 1886	+	2.0	511 520 529
	30	AB 1886	-	2.8	G G G T A T G G T G G C A G G C C C G
	41	AB 1157	+	13.8	-1
	45	AB 1157	+	2.8	
-1 deletion of G 389 or 390	32	AB 1157	+	8.8	381 390 399
					T C T A C G C C G G A C G C A T C G T
-2 deletion of GC 435-436 or 437-438 or CG 436-437	34	AB 1157	+	6.6	428 446
	36	AB 1157	+	8.8	G T T G C T G G C G C C T A T A T C G
					-2
Addition of a C within sequence 526-528	36				518 +1 C 536
-2 deletion of GC 548-549 or 550-551 or CG 549-550	33	AB 1157	+	13.8	G T G G C A G G C C C G T G G C C G G
					540 558
-1 deletion of G 416 and double transition at 414-415	35	AB 1157	+	8.8	A C T G T T G G G C G C A T C T C C
					406 424
					C A T C A C C G G C G C C A C A G G T
					double transition -1 A T

The sequences in the table are the wild-type sequences with the numbering defined by Sutcliffe¹⁵. The bases involved in the deletion mutations are boxed with dotted lines. The different possibilities to obtain a given mutated sequence are shown. Mutant 36 exhibits two mutations: a -2 deletion as in mutant 34 and a +1 addition of a C residue within the sequence CCC at positions 526-528. Mutant 35 has got a -1 deletion of a G at position 416 and a double transition, GC→AT, at position 414-415.

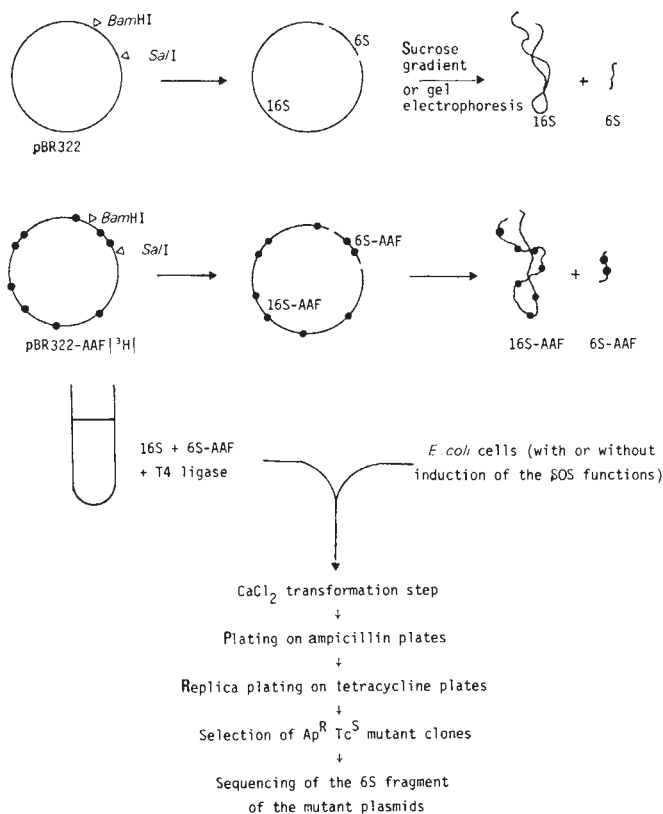


Fig. 1 Strategy for the site-directed mutagenesis experiments. The *E. coli* strains used were AB 1157 or AB 1886 (ref. 18). N-AcO-AAF (³H ring) was synthesized as described previously¹⁹ (specific activity 196 mCi mmol⁻¹). N-AcO-AAF (³H ring) reaction with supercoiled plasmid DNA was performed in 10 mM Tris, 1 mM EDTA, pH 8 (TE) buffer containing 5% of ethanol (DNA concentration 50 µg ml⁻¹). Removal of unbound fluorene derivatives was achieved by four successive ethanol precipitations. The number of AAF residues bound per plasmid molecule was determined as previously described²⁰. Samples of pBR322 reacted with N-AcO-AAF to various extents (ranging from 0 to 2.5% of modified bases) were digested with *Bam*HI and *Sal*I restriction enzymes (Boehringer, Mannheim). The large (16S) and the small (6S) fragments were separated and purified either by velocity sedimentation on sucrose gradients (5–20%) or by electrophoresis on 0.8% agarose or on 8% polyacrylamide gels followed by electroelution. T4 DNA ligase (Biolabs) was used to ligate the unmodified 16S fragment with either the unmodified 6S fragment or the 6S fragments obtained from the various AAF-modified pBR322 samples (6S-AAF). The ligation was performed in the conditions specified by the T4 DNA ligase manufacturer. The DNA fragment concentrations were 16.5 µg ml⁻¹ for the 16S fragment and 7.5 µg ml⁻¹ for the 6S fragment. UV irradiation of the cells before transformation: In some cases, the *E. coli* cells were UV irradiated before the transformation procedure to induce the cellular SOS response. The cells were UV irradiated as a suspension in 0.01 M MgSO₄ with a germicidal lamp (15 W, Phillips) at a dose giving about 50% survival (that is, 60 J m⁻² for the wild-type strain, AB 1157; 6 J m⁻² for the *uvrA* strain, AB 1886). The cells were then incubated in LB medium for 30 min at 37 °C to allow expression of the SOS functions. *E. coli* transformation and selection of the ampicillin-resistant (Ap^R) and tetracycline-sensitive (Tc^S) clones: The *E. coli* were made competent for transformation by the classical CaCl_2 treatment procedure²¹. The different ligation mixtures were diluted by a factor of 100 in (10 mM Tris), 10 mM CaCl_2 and 10 mM MgCl_2 (pH 7) and used to transform the competent cell suspension by mixing 1 vol of the DNA solution with 2 vol of the concentrated *E. coli* suspension. Following the transformation procedure, the cells were spread on LB plates containing ampicillin (50 µg ml⁻¹) and incubated at 37 °C overnight. The clones were then replated on LB plates containing tetracycline (20 µg ml⁻¹). Clones growing on Ap but not Tc were scored as $\text{Ap}^R \text{ Tc}^S$ mutants. Such individual mutant clones were then grown further in LB medium plus ampicillin for preparation of the plasmid DNA contained in these clones. Plasmid DNA was purified either on a small scale (10 ml of culture) by an adaptation of the method of Clewell and Helinski²² or on a larger scale (11 culture) by a NaCl/SDS lysis procedure followed by a CsCl/ethidium bromide centrifugation step²³. DNA sequence analysis of the mutants: Class II mutant plasmids (see text for the definition) were digested with *Bam*HI and *Sal*I restriction enzymes and ³²P-end-labelled at their 5' extremities with T4 DNA kinase (Boehringer, Mannheim). Strand separation and sequencing were performed according to the method of Maxam and Gilbert¹⁴.

frequency, provided the cells are exposed to UV before the transformation step. The mutant DNAs isolated from such experiments fall into two classes when analysed by gel electrophoresis: class I mutants defined as in the control experiment, and class II mutants, exhibiting the original size of pBR322 and retaining both *Bam*HI and *Sal*I restriction sites.

The frequency of class II mutants increases with the level of AAF modification and reaches about 3% at the highest level tested (Fig. 2). Note that only the class II mutation frequency is (1) a function of AAF modification and (2) dependent on UV irradiation of the host cell (for the conditions, see Fig. 1 legend). Both these properties indicate that class II mutants, as opposed to class I mutants, are actually induced by AAF adducts and that they require efficient expression of the cellular SOS functions. We, however, also obtained class II mutants, but at a lower frequency, in conditions where the SOS functions were not induced with UV light, tending to support the hypothesis that there is a constitutive level of error-prone repair¹³.

Class II mutants can also theoretically arise from 'non-targeted' mutations involving the SOS repair functions—mutations could occur during replication of the plasmid by the 'error-prone' SOS system, whether or not the plasmid has been chemically modified. In this hypothesis, however, the mutation could take place anywhere along the tetracycline gene and not exclusively in the 6S fragment. In fact, we have not found class II mutants in the pool of transformants obtained in the control experiment (that is, ligation of a non-modified 6S fragment).

Nine class II mutant plasmids were isolated from either the wild-type *E. coli* strain, AB 1157, or the corresponding *uvrA* mutant strain, AB 1886. The double *Bam*HI–*Sal*I-digested DNA was ³²P-end-labelled at the 5' extremities and sequenced according to the Maxam and Gilbert technique¹⁴. The sequence of the wild-type 6S fragment of pBR322 was found to be identical to that published by Sutcliffe¹⁵.

In all of the nine class II mutants we found a mutation located within the 6S fragment. All the mutants showed a deletion of either a single G · C base pair or a doublet of adjacent G · C · G base pairs. Two of these mutants (numbers 35 and 36) also had a second mutation (Table 1). Mutants 34 and 36 both exhibit a –2 deletion within the alternating GCGC sequence at positions 435–438 (deletion of a GC sequence at position 435–436 or 437–438 or a CG sequence at position 436–437). (Numbering starts clockwise from the unique *Eco*RI restriction site.) Moreover, mutant 33 also exhibits a –2 deletion within a GCGC sequence at positions 548–551. Note that the same 6-nucleotide long sequence, GGCGCC, occurs at the site of mutations 34, 36 and 33. The fact that this mutation has been observed three times suggests that the given sequence is a good candidate for a mutational hot spot. Such a GC deletion in an alternating GC

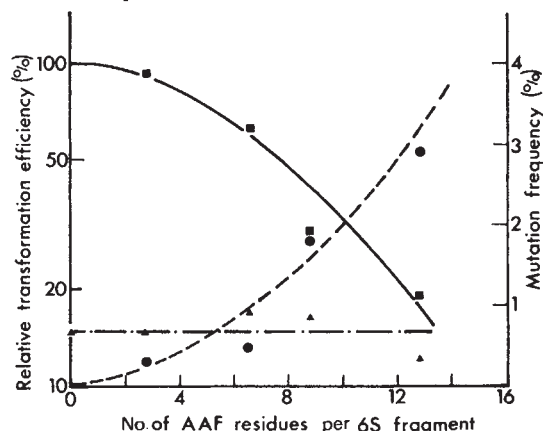


Fig. 2 Relative transformation efficiency and mutation frequencies as a function of the number of AAF residues bound to the 6S fragment. The transformation of *E. coli* AB 1157 strain has been performed after UV irradiation of the cells (60 J m⁻²) as described in Fig. 1 legend. Selection of the transformants was done on LB plates containing ampicillin (50 µg ml⁻¹). Mutants were selected by replica plating on LB plates containing tetracycline (20 µg ml⁻¹). ■, Relative transformation efficiency (log scale); ▲, class I mutants; ●, class II mutants.

sequence was shown *in vivo* to be a hot spot for reversion of the mutation, *his D3052*, in *Salmonella* by the mutagen 2-nitrosofluorene¹⁶. It is striking that the same type of mutation occurs in both a reversion and forward mutation assay.

As shown in Table 1, four (4, 30, 41 and 45) of the nine mutants show a deletion of a single G residue at position 520 or 521. Note that the four mutants have arisen in different conditions (in two different strains, with or without UV induction of the SOS functions, with very different AAF modification levels). The reason why this particular sequence is a hot spot for mutagenesis is not clear. The possibility that it is a hot spot for the AAF binding reaction itself (the premutational event) is being investigated. Alternatively, it is possible that the processing of the premutational lesions (by the excision repair enzymes for example) is strongly sequence dependent, in which case a hot spot for mutagenesis could arise from the inability of the error-free repair enzymes to excise the lesion at the given site.

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The mechanism by which single GC base pair deletion mutations occur can be tentatively explained on the basis of the insertion-denaturation model^{7,10}, whereby the guanine modified at C-8 with the AAF residue is flipped outside the double helix while the AAF residue is inserted between the two neighbouring base pairs. During replication, the AAF-modified guanine, an essentially non-coding base, blocks the replication machinery¹⁷. One can hypothesize that SOS functions then become involved at the replication fork which permit this block to be bypassed by allowing the replication to restart at the next base, thus giving rise to the deletion of the AAF-modified guanine residue.

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Mechanism of *E. coli* RecA protein directed strand exchanges in post-replication repair of DNA

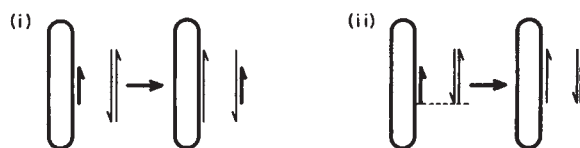
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Escherichia coli mutants carrying *recA*[−] are both recombination deficient and unable to perform post-replication repair^{1,2}. The product of the *recA* gene regulates the inducible DNA repair functions (the SOS response to DNA damage)^{3,4}, and is directly involved in homologous pairing^{5–8} and strand exchange^{9–12}, two reactions fundamental to recombination and post-replication repair. The filling of post-replication gaps is thought to occur by homologous pairing of the gapped DNA duplex with an intact duplex, followed by cutting of the intact molecule so that sister strand exchanges can take place. Using *in vitro* systems, we have shown previously that purified RecA protein binds cooperatively to duplex DNA that contains gaps¹³, and promotes joint molecule formation (synapsis) between gapped and intact duplexes^{7,8}. Moreover, RecA protein promotes a reciprocal exchange of strands between paired DNA molecules^{10,12,14}. Here, we investigate the mechanism of sister strand exchange thought to occur during post-replication repair. We show that RecA protein initiates strand exchange from a nicked duplex, transferring the 3'-OH terminus at the nick into the single-stranded (ss) region of the gapped molecule. In the presence of ATP, two heteroduplex molecules are formed as RecA protein drives the reciprocal exchanges in one direction starting at the site of the original crossover.

The assay for strand exchange involves the incubation of ³²P-labelled duplex restriction fragments with unlabelled circular ssDNA, to which we have annealed a short complementary ³H-labelled fragment (Fig. 1a(i)). The annealed substrate

a DNA substrates that initiate strand exchange



b DNA substrates that do not exchange strands

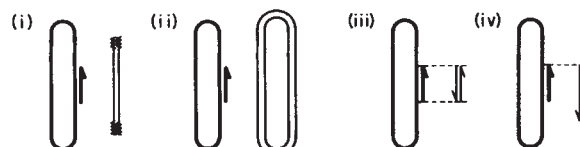


Fig. 1 DNA substrates used in RecA protein-mediated strand exchange tests. The 3' terminus of each strand is indicated with a half arrow. *a*(i), Annealed substrate (ΦX (+)ssDNA with a 503-nucleotide linear duplex (872-base pair (bp) *HaeIII* fragment 3) that overhangs both ends of the annealed fragment. These substrates were used in the experiment of Fig. 2. (ii), Homologous duplex that overhangs one end of the annealed fragment (Fig. 2d in ref. 14). Exchange is dependent on polarity and is observed only if the overhanging (−)strand of the duplex has a 3' terminus¹⁴. *b*, Strand exchange does not occur when: (i), the duplex has non-homologous ends (wavy lines) (Fig. 2b in ref. 14); (ii), the duplex has no free end (Fig. 1 V in ref. 10); (iii) the duplex is flush-ended with the annealed fragment (Fig. 1 IV in ref. 10); (iv), the (−)strand of the one overhanging end of the duplex has a 5' terminus (Fig. 2c in ref. 14).

sedimented faster through neutral sucrose than the short duplex fragments (Fig. 2a). However, incubation in the presence of RecA protein caused an exchange of labelled strands, heteroduplex DNA being formed as 57% of the ³H-labelled annealed fragments were transferred from the ssDNA in exchange for ~50% of the (−)strands of the duplex (25% of total ³²P label). These results, shown in Fig. 2b, indicate that RecA protein promotes reciprocal strand exchanges to form two heteroduplex molecules and are described in more detail elsewhere^{10,14}.

We wished to test two aspects of RecA protein-mediated strand exchange in relation to the proposed mechanism of post-replication repair¹⁴. First, does RecA protein initiate strand exchange at a nick in an intact duplex, so transferring a complementary strand into the single-stranded region of a homologous gapped duplex, and second, does RecA protein drive the reciprocal strand exchange in a polar way from the site of the initial crossover. To test these points we constructed the DNA substrates shown in Fig. 3 (i) and (ii).

In a control experiment we reacted the substrates shown in Fig. 3 (i) with RecA protein. The annealed substrate is the same as that used in Fig. 2; it contains both duplex and single-stranded regions and is therefore similar to a gapped duplex. The linear duplex has one end flush with the *AvaI* end of the annealed fragment and the opposite end is non-homologous with Φ X DNA. We have shown previously^{10,14} that duplexes with ends non-homologous or flush with the annealed fragment do not initiate strand exchanges (Fig. 1b (i) and (iii)). As expected with a duplex of this structure, strand exchanges were not observed (Fig. 3a).

The experiment was then repeated using a similar linear duplex that had been nicked at the *Bam*HI site in one or the other of the two strands (indicated with an arrow in Fig. 3 (i)). Assuming that the site-specific nicking occurred with the same efficiency in both strands, we estimated that ~20% of the linear fragments used in the reaction mixture contained nicks in the (–)strands. We therefore used an excess of linear duplexes in these experiments. Efficient strand exchanges took place, 41% of the annealed fragments being transferred (Fig. 3b).

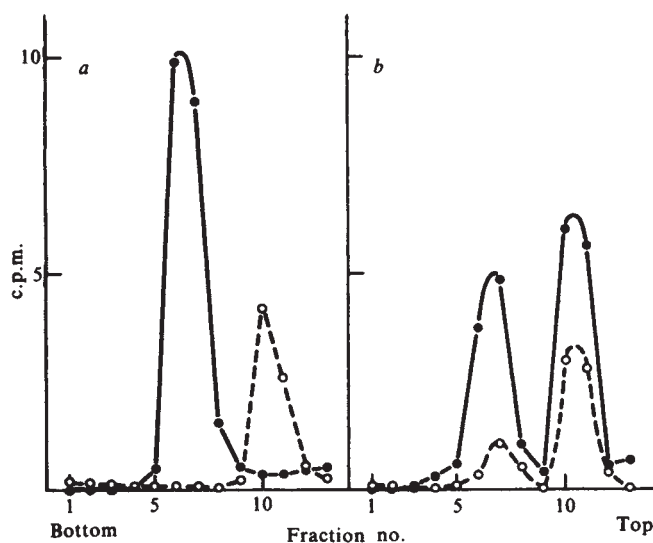


Fig. 2 Neutral sucrose sedimentation profiles showing strand exchanges between the DNA molecules of Fig. 1a(i). Sedimentation is to the left. *a*, Annealed substrate (unlabelled Φ X ssDNA with ³H-labelled *AvaI*–*AvaII* annealed fragment) with ³²P-labelled Φ X *HaeIII* 3 linear duplex; *b*, same as *a* but with RecA protein. Annealed substrate, duplex fragments and RecA protein were added where indicated at 1.75 μ M, 0.42 μ M and 5 μ M, respectively. Duplex fragments and the annealed substrate were prepared as described previously¹⁰ except that *AvaI* and *AvaII* were used to digest the ³H-labelled Φ X RFI DNA for annealed substrate preparation. RecA protein was purified as described elsewhere¹³. The standard reaction mixture contained in a total volume of 100 μ l, 20 mM Tris-HCl pH 7.5, 25 mM MgCl₂, 2 mM dithiothreitol, 2 mM ATP and 100 μ g ml^{–1} bovine serum albumin. Incubation was for 45 min at 37 °C. The reaction was stopped by the addition of 50 mM EDTA, and proteins were removed by treatment with 200 μ g ml^{–1} proteinase K and 1% SDS¹⁰. Centrifugations were through 5 ml of 5–20% neutral sucrose in a Beckman SW 50.1 rotor at 45,000 r.p.m. for 180 min at 4 °C. Fractions collected from the gradients were counted for radioactivity in Formula 963 scintillation fluid (NEN). ●, ³H, $\times 10^{-2}$; ○, ³²P, $\times 10^{-1}$.

Table 1 Tests for strand exchange between the annealed substrate and a nicked duplex: the effect of nick closing by T4 DNA ligase

Linear duplex	Ligase	RecA	% Strand exchange
<i>a</i> , Nicked chimaeric duplex (Fig. 3 (i))	–	+	33
<i>b</i> , Same as <i>a</i>	+	+	8
<i>c</i> , Homologous duplex (Φ X <i>HaeIII</i> – <i>AvaI</i>)	–	+	40
<i>d</i> , Same as <i>c</i>	+	+	40

The reactions described here were carried out in two stages, ligation and then strand exchange. In the first, the duplex fragments, either 0.42 μ M Φ X linear *HaeIII*–*AvaI* or 3.15 μ M nicked pSW1 *HaeII*–*AvaI*, were incubated for 20 min at 25 °C with or without 8×10^{-3} units of T4 DNA ligase (BRL) in 100 μ l of 50 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 2 mM dithiothreitol, 1 mM ATP and 50 μ g ml^{–1} bovine serum albumin. The reaction mixtures were heated for 5 min at 60 °C to inactivate the ligase, and cooled to ice temperature. In the second stage, we added 100 μ l of buffer containing 20 mM Tris-HCl pH 7.5, 25 mM MgCl₂, 2 mM dithiothreitol, 2 mM ATP, 100 μ g ml^{–1} bovine serum albumin, 1.75 μ M of the annealed substrate (Fig. 1a (i)) and 5 μ M RecA protein, and incubated the mixtures for 45 min at 37 °C. Proteins were removed from the DNA and the reaction products sedimented through neutral sucrose as described in Fig. 2 legend. The linear duplex Φ X *HaeIII*–*AvaI* was prepared as described previously¹⁴ and the nicked duplex was prepared as in Fig. 3 legend. The per cent strand exchange was calculated from the amount of total ³H-labelled annealed fragments that were displaced from the annealed substrate. The concentration of DNA ligase used here was predetermined as an amount sufficient to ligate 80% (as determined by alkaline sucrose sedimentation) of the nicks in 100 μ l of 3.5 μ M pBR322 DNA. This DNA had been nicked by pancreatic DNase I in the presence of ethidium bromide. The conditions of the experiment did not result in any dimerization of the linear duplex fragments (detected by polyacrylamide gel electrophoresis).

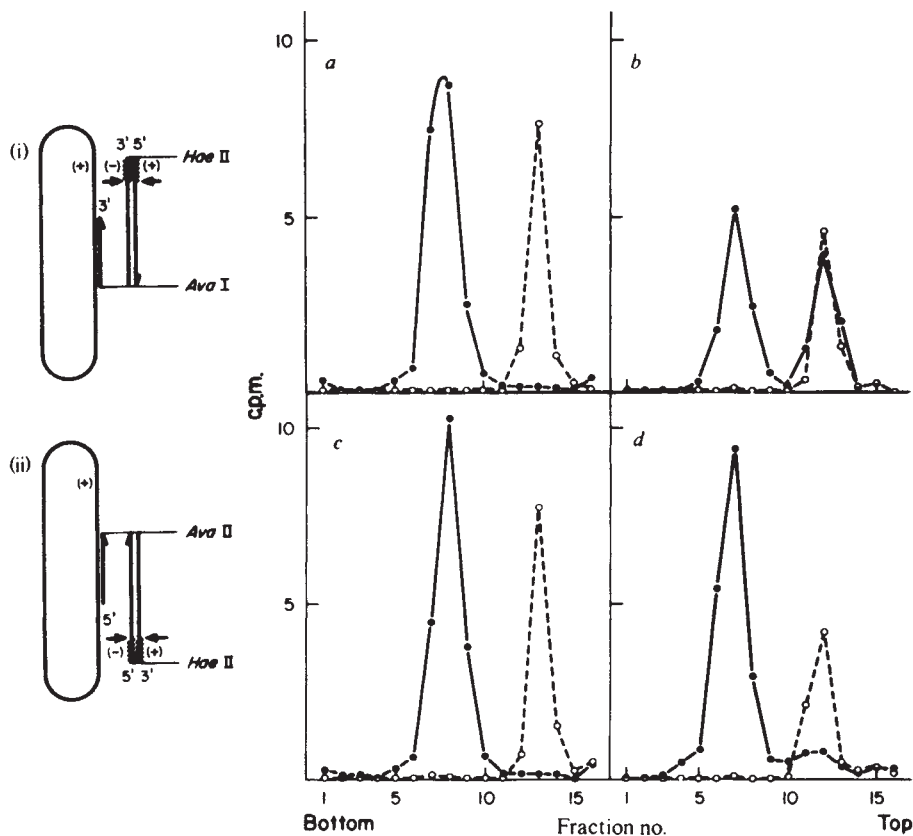
To confirm that strand exchanges were indeed initiated from the nick in the duplex molecule, we incubated the nicked DNA with T4 DNA ligase just before the addition of RecA protein and the annealed substrate to the reaction mixture. RecA protein did not promote strand exchanges when the nicks were preligated (Table 1b), whereas incubation of an identical reaction mixture without DNA ligase resulted in efficient strand exchanges (Table 1a). In a control to test whether RecA-mediated strand exchanges might be inhibited by the presence of DNA ligase, we examined the effect of ligase on a similar reaction between the annealed substrate and a linear duplex. The results in Table 1c and d show that strand exchanges occurred with the same efficiency in the absence or presence of DNA ligase, indicating that no inhibitory factors were present in the reaction mixture.

These results show that strand exchanges take place efficiently between an annealed substrate and a duplex nicked at the *Bam*HI site (Fig. 3 (i)). The nick was essential for the initiation of strand exchanges.

It is interesting that the 3'-terminal base of the strand transferred from the nicked duplex is derived from a molecular linker inserted into the plasmid pSW1 during construction of the chimaeric plasmid¹⁴. The terminus ending CCG-3'-OH is transferred to the complementary Φ X sequence ...GGT...5' to form a G/T single nucleotide mismatch (sequence from Sanger *et al.*¹⁵). Thus, although this single nucleotide mismatch at the 3' terminus does not block strand exchange, we have shown previously that a non-homologous terminal region of 150 bases is sufficient to block the reaction¹⁴.

To test whether strand exchanges from a nick are driven in a polar way, we incubated the DNA substrates shown in Fig. 3 (ii) with RecA protein. These substrates are similar to those described above (Fig. 3 (i)) except that the *Bam*HI nick of the duplex is in a different position relative to the annealed fragment. The results shown in Fig. 3d indicate that this nicked duplex is inefficient at strand exchange, ~9% of the ³H-labelled annealed fragments being transferred. In the absence of a nick,

Fig. 3 Neutral sucrose sedimentation profiles to show initiation of strand exchange by the nicked duplexes shown in (i) and (ii). *a*, Annealed substrate with intact *HaeII*-*AvaI* duplex DNA and RecA protein. *b*, Same as *a* but the duplex was nicked at the *Bam*HI site. *c*, Annealed substrate with intact *AvaII*-*HaeII* duplex DNA and RecA protein. *d*, Same as *c* but the duplex was nicked at the *Bam*HI site. In substrates (i) and (ii), non-homologous DNA is indicated by a wavy line, and the site of nicking by *Bam*HI/EtBr is arrowed. In all reactions, the annealed substrate, duplex fragments and RecA protein were present at 1.75 μ M, 3.15 μ M and 5 μ M, respectively. Incubation and sedimentation were as described in Fig. 2 legend. Duplex fragments were prepared by digesting pSW1 form I 32 P-labelled DNA with *HaeII* and fragment 3 was purified¹⁴. The fragment was extracted with isobutanol to remove ethidium bromide and digested with either *AvaI* or *AvaII*. The larger fragments in each case were purified and extracted with phenol and isobutanol. Nicked duplex fragments were prepared in the same way from pSW1 DNA that was nicked by *BamI* in the presence of ethidium bromide²¹. As 80% of the closed circles were nicked in the *Bam*HI/EtBr reaction (determined by agarose gel electrophoresis and alkaline sucrose sedimentation), and as the single nick could be in either strand at one of the two *Bam*HI sites, we estimate that 20% of the duplexes contained nicks in the (-) strand at the *Bam*HI site. ●, ^3H , $\times 10^{-2}$; ○, ^{32}P , $\times 10^{-2}$.



we found the normal background (<5%), indicating that no strand exchange had taken place (Fig. 3c). Comparison of the data presented in Fig. 3b and d indicates that RecA protein shows a strong directional bias for the extension of the heteroduplex region from the nick. As the polarity of each DNA strand is known from the DNA sequence¹⁵, the directionality of

RecA-driven exchanges can be determined. The efficient strand exchange initiated by the nicked duplex (Fig. 3 (i)) occurs by transfer of the free 3'-OH terminus at the nick of the complementary strand into the single-stranded region of the annealed substrate. The heteroduplex region is then extended by RecA protein transferring strands in a 3' to 5' direction (relative to the transferred strand). The polarity of strand exchange shown here is therefore similar to those involving exchanges initiated by a linear duplex^{14,16,17}.

The experiments described here suggest a way in which RecA protein may exchange DNA strands in post-replication repair. As shown in Fig. 4, DNA polymerase moving in a 3' to 5' direction along the template strand is blocked at a pyrimidine dimer and reinitiates synthesis further along the chain. The daughter strand is therefore terminated with a 3'-OH at the position of the dimer (Fig. 4a). The resulting ssDNA activates the RecA protease¹⁸, triggering the SOS response and increasing the synthesis of RecA protein in readiness for pairing with the intact sister duplex (Fig. 4b). Pairing may occur at the site of the gap with the single-stranded region of the gapped molecule wound in the major groove of the duplex. Once homologous contacts are established, a cutting *in trans* enzyme may nick the intact sister duplex at a site opposite the gap¹⁹ (Fig. 4b). The nick enables RecA protein to transfer the 3'-OH terminus of the nicked strand into the gap, thus providing an intact strand complementary to the one containing the dimer (Fig. 4c). The strand exchange reaction is therefore initiated in a three-stranded way as RecA protein drives branch migration and heteroduplex formation beyond the dimer. The 3'-OH terminus of the gapped strand is also transferred, producing a crossed strand exchange (Fig. 4d). From that point on, the four strands are paired and exchanged as described previously¹⁰ to produce duplex molecules. Both helices can now be repaired by DNA polymerase I and ligase (Fig. 4e) and completion of repair permits the release of RecA protein and terminates the SOS response. The resulting crossed strand exchange is presumably

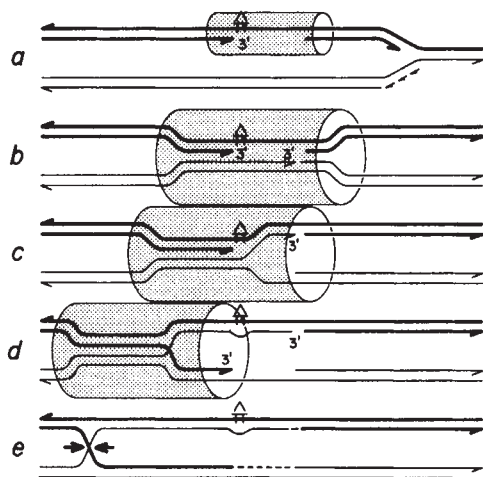


Fig. 4 Model for the repair of a post-replication gap by RecA protein-mediated sister strand exchanges. RecA protein molecules (shaded area) bind at the post-replication gap (a) promoting homologous pairing (b). The intact molecule is nicked by the cutting *in trans* activity (b) and the 3' terminus of the nicked strand is transferred by RecA protein into the post-replication gap (c). Directional strand transfer driven by RecA protein results in the reciprocal transfer of the 3' terminus of the gapped strand to produce a crossed strand exchange or Holliday structure (d). The crossed strand exchange is cut leading to completion of the repair (e).

resolved by a further recombination enzyme, perhaps similar to the phage T4 gene 49 nuclease²⁰. The final structure retains the pyrimidine dimer, but now has an intact complementary strand and can be repaired by excision enzymes.

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Effect of base-pair stability on the melting of superhelical DNA

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It is well established that the structure^{1,2} as well as the stability of the DNA duplex is affected by supercoiling. Supercoiled DNA has been shown to possess increased stability and is thus characterized by an elevated melting temperature as well as a broader melting transition. Thermal melting analysis has been the most widely used method for studying the structural and dynamic properties of complex DNA species. Here we report a remarkable attribute of supercoiled DNA, that the width of the melting transition of superhelical DNA does not depend appreciably on the relative stabilities of its A·T and G·C base pairs.

It has been shown earlier^{3,4} that superhelical DNA (DNA I) melts at higher temperatures and over a broader temperature range than do open circular molecules (DNA II). These experiments were carried out in conditions such that the stabilities of the A·T and G·C base pairs were substantially different. To eliminate the possible influence of this factor on the melting process, we performed the thermal denaturation of DNA in experimental conditions in which the A·T and G·C base pairs were of equal stability by dissolving the DNA in a concentrated solution of tetraethylammonium bromide (TEA)^{5,6}.

Figure 1a shows the integral and differential melting curves of the replicative form of phage ΦX174 DNA in a solution containing 3 M TEA (the sample was a 10:1 mixture of DNA I and DNA II). The step at 58 °C in the melting curve (the sharp peak in the differential curve) corresponds to the melting of

DNA II. Its melting range is less than 1 °C wide, thus indicating that the thermal stabilities of A·T and G·C base pairs are approximately equal^{5,6}.

The melting of DNA I seems to be quite different. Its first intriguing feature is the width of the denaturation range. Melting is appreciable at 60 °C but even at 95 °C ~10% of the base pairs are still in the native state. Over the temperature range 60–90 °C the derivative $d\theta/dT$ increases and reaches 1/25 °C at the transition midpoint ($\theta = 1/2$).

Figure 1b shows the melting curves of the same sample as in Fig. 1a, but in a solution containing 7.3 M NaClO₄ (conditions in which the difference in stability between A·T and G·C base pairs, $T_{GC} - T_{AT}$ is ~56 °C)⁷. Here DNA II melting occurs over a broad (8–10 °C) range, and the differential curve shows a resolved fine structure.

In contrast, the melting profile of DNA I remains practically unchanged, and the derivative $d\theta/dT$ at the transition midpoint has the same value of 1/25 °C. Moreover, the temperature difference between the melting midpoints of DNA I and DNA II is the same (25 °C) in both experimental situations (Fig. 1a, b). These findings elucidate the fundamental property of superhelical DNA—that, in contrast to linear DNA or DNA II, its melting is insensitive to the value of $T_{GC} - T_{AT}$ and, therefore, to the nucleotide composition heterogeneity.

A slight increase of absorbance before the melting of DNA II can be seen in both Fig. 1a and b. In Fig. 1a this effect is not superimposed on the melting of DNA II and its magnitude can be evaluated ($\theta \approx 0.05$); it corresponds roughly to the degree of denaturation necessary to remove all superhelical turns (as reported elsewhere⁸ the superhelical density of ΦX174 DNA is 0.063). Thus, the increase in absorbance could be attributed to early melting of DNA I³. However, this effect is of the same order of magnitude as the baseline instability of the instrument, further investigation is necessary.

The theory of the 'helix-coil' transition in polynucleotides without topological constraints (linear or open circular DNAs) is

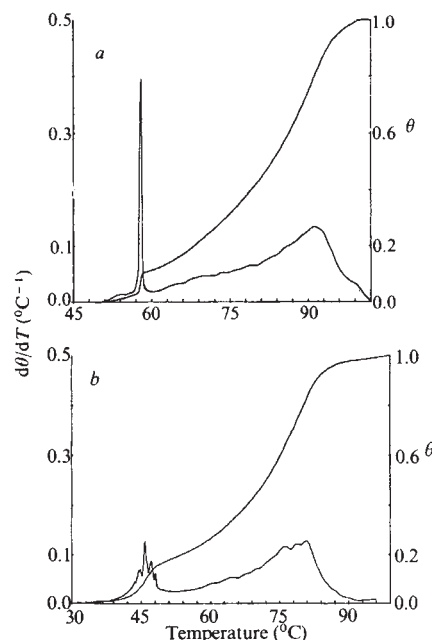


Fig. 1 The integral and differential melting curves of the replicative form of phage ΦX174 DNA; a, in the presence of 3 M TEA ($T_{GC} - T_{AT} = 0$ °C)^{5,6}, b, in the presence of 7.3 M NaClO₄ ($T_{GC} - T_{AT} = 56$ °C)⁷. A 120-μl aliquot of solution contained 10–12 μg ml⁻¹ DNA (10:1 mixture of DNA I and DNA II), 10 mM phosphate buffer pH 8.0 and the concentration of TEA or NaClO₄ indicated above. The melting curves were registered on a Carry 218 spectrophotometer, equipped with a specially constructed sample holder with a facility for temperature control. Temperature scanning rate was 0.2 °C min⁻¹. The melting curves were differentiated using a Hewlett-Packard 9830A computer.

well developed, and excellent agreement with experimental data has been achieved for such molecules⁷. However, the above-mentioned features of the melting of covalently closed DNA cannot be explained within the framework of this theory. An adequate explanation should be based on a correct account of the effect of topological constraints on the thermodynamical properties of DNA.

Despite the disruption of the double helix, the topological state of DNA I (linking number of the strands, Lk) remains invariant. That leads to an excess of Lk and therefore to extra free energy in DNA I compared with DNA II at the same degree of denaturation ($\Delta Lk \sim \theta$).

When DNA is treated as a thin ribbon (an approximation which seems to be reasonable at small degrees of denaturation), a part of Lk can be isolated (writhing, Wr) which is determined by the spatial configuration of DNA duplex axis only⁹. Then the difference ($Lk - Wr$) will account for the twist (Tw) of the ribbon⁹. The extra free energy of DNA I within the melting interval is a function of both Wr and Tw . These values cannot be precisely estimated at present. In this situation a heuristic approach seems to be reasonable. In the most advanced theoretical work on the problem¹⁰ it was assumed that the strands in the melted region remain twisted to the same extent as before denaturation. Thus, the possible contribution of Wr to Lk was ignored. According to this model, the melting range for DNA I should be 2–3 times larger than that for DNA II¹⁰. The latter factor fits the experimental data when the value of $T_{GC} - T_{AT}$ is large. However, for $T_{GC} \approx T_{AT}$ there is marked disagreement between calculated and experimentally obtained values for the width of melting range.

We consider the failure of the model to be due to the neglect of the possible contribution of Wr to Lk . Because of the enhanced flexural 'softness' of partially melted DNA the latter can be significant. The theoretical treatment of the model, allowing for such a possibility, will be reported elsewhere.

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Polycythaemia- and anaemia-inducing strains of spleen focus-forming virus differ in post-translational processing of envelope-related glycoproteins

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A virus preparation was isolated by Friend which produced a rapid erythroblastosis in certain strains of mice¹. Although the original virus preparation induced a slight anaemia in the terminal stages of the disease, other variants, derived from various *in vivo* and *in vitro* passages of the original virus, were associated with polycythaemia^{2,3}, and the different viruses were accordingly classified as FVA (Friend virus anaemia-inducing) or FVP (Friend virus polycythaemia-inducing). Another important difference between the two strains is that whereas FVP induces in infected mice cells that are capable of forming

colonies *in vitro* in the absence of erythropoietin^{4,5}, colonies formed by cells derived from FVA-infected mice are erythropoietin dependent^{6–9}. Differences were also noted in the amount of haemoglobin present in erythroid bursts produced after *in vitro* infection of haematopoietic cells with FVP or FVA²². Both FVA and FVP virus stocks have been shown to consist of at least two different viruses: the spleen focus-forming virus (SFFV_P or SFFV_A), a replication defective virus; and the Friend murine leukaemia virus (F-MuLV), a replication competent virus which acts as a helper virus for replication of the defective SFFV^{6,23}. Here, in an attempt to correlate differences in the biological effects of the SFFVs derived from FVP and FVA stocks with biochemical properties of proteins encoded by them, we have compared the post-translational processing of the envelope glycoproteins encoded by these viruses, and have found both qualitative and quantitative differences in the processing of the envelope glycoproteins of the two virus stocks.

Although the genomes of the SFFVs derived from FVP and FVA stocks differ slightly¹⁰, both encode similar primary translational products—a 45,000 molecular weight (M_r) gag-related protein (p45^{gag}) and a 52,000- M_r envelope-related glycoprotein (gp52^{env})^{6,11,12}. Studies with subgenomic fragments of molecularly cloned SFFV_P have shown that the biological activity of SFFV requires¹³ the gene encoding gp52^{env}, but not that encoding p45^{gag}. Differences in the tryptic peptides of gp52^{env} exist among different strains of SFFV_P (S.K.R., unpublished data) as well as between the SFFVs derived from FVP and FVA stocks⁶. However, these differences have not been correlated with the different biological effects of SFFV_P and SFFV_A.

When NRK fibroblasts were nonproductively infected with the Lilly-Steeves strain of SFFV_P (ref. 14) and the major product of the *env* gene detected by pulse-labelling the cells with ³⁵S-methionine and immune precipitating with specific anti

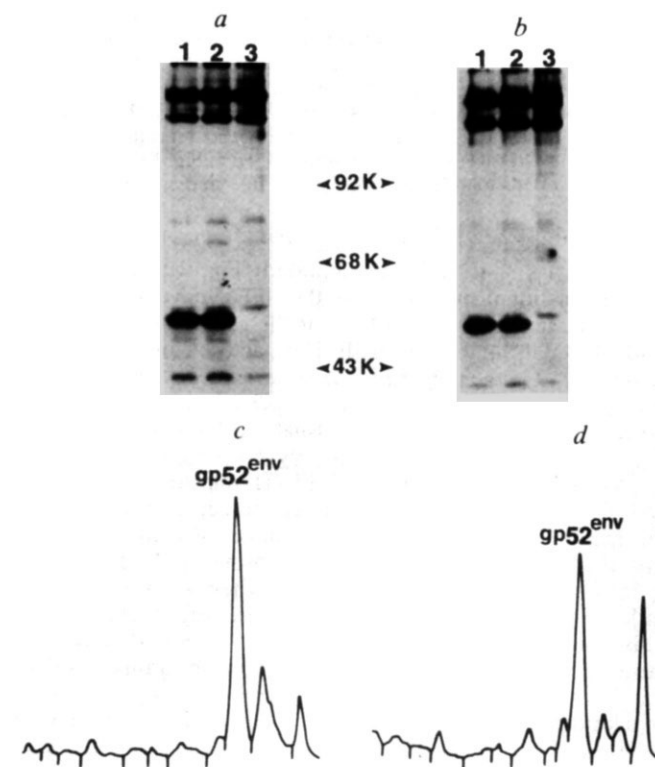


Fig. 1 Immune precipitation of ³⁵S-methionine-labelled SFFV_P and SFFV_A cytosol proteins. Fibroblasts nonproductively infected with either SFFV_P (a) or SFFV_A (b) were labelled for 30 min with ³⁵S-methionine and then immune precipitated with goat anti-Rauscher MuLV gp70 serum (lanes 1), a goat antiserum specific for the gp70 of MCF murine leukaemia viruses (lanes 2), or normal goat serum (lanes 3). Precipitates were then electrophoresed on 7% SDS-polyacrylamide gels and exposed to X-ray film as previously described¹². c, d Represent densitometer scans of lanes 1 in a and b, respectively.

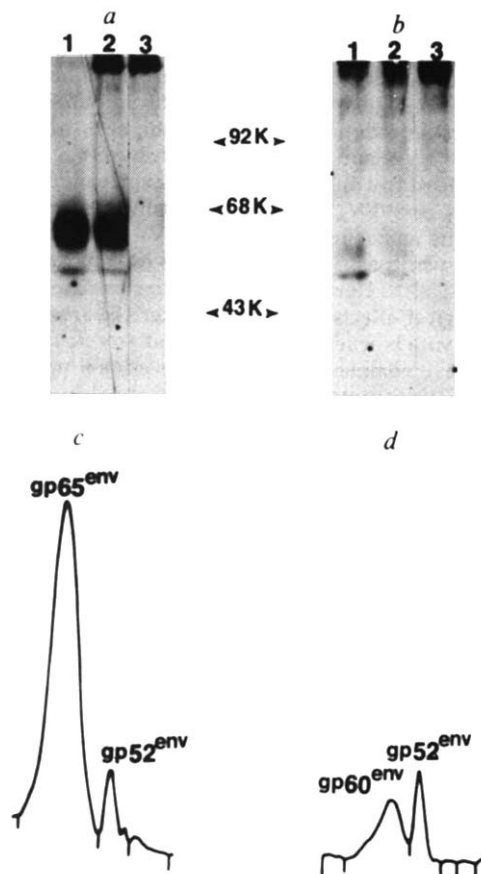


Fig. 2 Immune precipitation of ^3H -galactose-labelled SFFV_P and SFFV_A cytosol proteins. Fibroblasts nonproductively infected with either SFFV_P (a) or SFFV_A (b) were labelled with ^3H -galactose and then immune precipitated with goat anti-Rauscher MuLV gp70 serum (lanes 1), a goat antiserum specific for the gp70 of MCF murine leukaemia viruses (lanes 2), or normal goat serum (lanes 3). The precipitates were processed as described in Fig. 1 legend. c, d Represent densitometer scans of lanes 1 in a and b, respectively.

gp70 sera as previously described¹², a 52,000- M_r protein (gp52^{env}) was specifically precipitated (Fig. 1a, lanes 1, 2). Post-translational processing of the SFFV envelope proteins and expression of these proteins at the cell surface were then studied by pulsing the cells with ^3H -galactose¹⁵ or labelling the cells with ^{125}I in the presence of lactoperoxidase¹², respectively, before immune precipitation with specific antisera. Proteins precipitated in each case were visualized by autoradiography after electrophoresis on SDS-polyacrylamide gels. When SFFV_P/NRK cells were labelled with ^3H -galactose, gp52^{env} and 65,000- M_r glycoprotein (gp65^{env}) were detected, indicating that the gp52^{env} is processed by the addition of complex carbohydrates to a higher molecular weight form (Fig. 2a, lanes 1, 2). gp65^{env} was detected on the cell surface when cells were labelled with ^{125}I in the presence of lactoperoxidase (Fig. 3a, lanes 1, 2). This seems to be a common pathway for SFFV_P envelope proteins, as two other polycythaemia-inducing strains of SFFV, the Mirand and Axelrad strains, synthesize 62–68,000- M_r envelope glycoproteins which can be detected after metabolic labelling of nonproducer cells with ^3H -galactose¹⁵ or cell-surface labelling with ^{125}I (S.K.R., unpublished data).

Cells nonproductively infected with SFFV derived from an anaemia-inducing stock of Friend virus¹¹ also synthesized a 52,000- M_r glycoprotein that could be labelled with ^{35}S -methionine and immune precipitated with anti-gp70 sera (Fig. 1b, lanes 1, 2). When SFFV_A nonproducer cells were labelled with ^3H -galactose, gp52^{env} but no gp65^{env} was detected (Fig. 2b, lanes 1, 2). However, the cells contained a low amount of a 60,000- M_r glycoprotein that was precipitable with anti-gp70 sera. When we attempted to label surface proteins

with ^{125}I and lactoperoxidase, no envelope-related proteins were detected on the surface of SFFV_A nonproducer cells (Fig. 3b, lanes 1, 2).

The differences in the levels of processed envelope glycoproteins in SFFV_P and SFFV_A nonproducer cells were quantified by scanning films of the gels with a densitometer. Cells nonproductively infected with either expressed almost equivalent levels of gp52^{env} (Fig. 1, 2, panels c, d) but SFFV_P nonproducer cells expressed eightfold higher levels of any high molecular weight, galactose-containing envelope glycoprotein than did SFFV_A nonproducer cells (Fig. 2c, d).

Differences in processing of SFFV_P and SFFV_A gp52^{env} were also detected *in vivo*. Spleens of mice infected with either the FVP complex (SFFV_P pseudotyped with F-MuLV) or the FVA complex (SFFV_A pseudotyped with F-MuLV) were labelled with either ^{35}S -methionine or ^3H -galactose and immune precipitated with anti-gp70 sera. F-MuLV and SFFV envelope glycoproteins could be distinguished from each other using a MCF-specific antiserum that will precipitate SFFV, but not F-MuLV, envelope glycoproteins¹². As shown in Fig. 4, spleens from mice infected with either virus expressed equivalent amounts of SFFV gp52^{env} (Fig. 4a, lanes 1, 2; b, lanes 1, 2) but differed in the levels of the processed form of gp52^{env} detected by labelling with ^3H -galactose (Fig. 4c, lanes 1, 2; d, lanes 1, 2). Equivalent levels of ^{35}S -methionine- and ^3H -galactose-labelled F-MuLV-encoded envelope proteins were detected in spleens from animals infected with either virus.

Our results indicate that the processing of the envelope glycoproteins encoded by the SFFVs derived from FVP and FVA stocks differs both quantitatively and qualitatively. The gp52^{env} encoded by three different strains of SFFV_P is processed to a galactose-containing 65,000- M_r glycoprotein which eventually appears on the cell surface, whereas most of the gp52^{env} encoded by SFFV_A is not further processed to a higher molecular weight, galactose-containing form and cannot be detected on the cell surface. Cells nonproductively infected with the Rauscher strain of SFFV, another anaemia-inducing erythroblastosis virus¹⁶, show similar differences in envelope glycoprotein processing and transport.

A variety of membrane glycoproteins have been shown to contain a signal peptide at their amino terminus that is believed to be necessary for proper processing of the glycoproteins and transport to the cell surface^{17–19}. Although such a signal peptide has not been identified for any of the envelope proteins of RNA tumour viruses, one could postulate that the difference in the processing of SFFV_P and SFFV_A envelope proteins is due to a

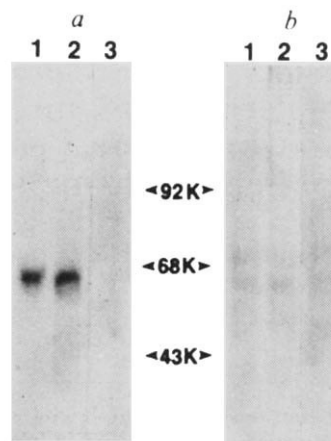


Fig. 3 Immune precipitation of ^{125}I -labelled SFFV_P and SFFV_A cell-surface proteins. The surface proteins of cells nonproductively infected with either SFFV_P (a) or SFFV_A (b) were labelled with ^{125}I in the presence of lactoperoxidase and then immune precipitated with goat anti-Rauscher MuLV gp70 serum (lanes 1), a goat antiserum specific for the gp70 of MCF murine leukaemia viruses (lanes 2), or normal goat serum (lanes 3). The precipitates were processed as described in Fig. 1 legend.

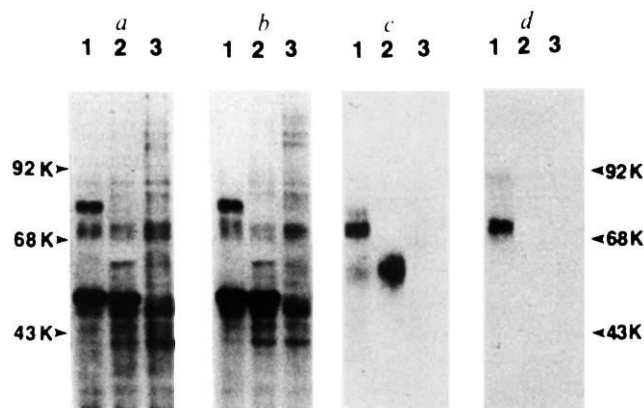


Fig. 4 Immune precipitation of spleen cell extracts from mice infected with either the FVP or FVA complex. Spleen cells from BALB/c mice infected 21 days previously with either 4×10^4 focus-forming units of F-MuLV/SFFV_P (a, c) or $1-2 \times 10^4$ focus-forming units of F-MuLV/SFFV_A (b, d) were labelled with either ^{35}S -methionine (a, b) or ^3H -galactose (c, d) and then immune precipitated with goat anti-Rauscher MuLV gp70 serum (lanes 1), a goat antiserum specific for the gp70 of MCF murine leukaemia viruses (lanes 2), or normal goat serum (lanes 3). Precipitates were processed as described in Fig. 1 legend.

mutation in the gene encoding the signal peptide of the SFFV_A gp52^{env}, resulting in defective processing and failure to transport the protein to the cell surface. Consistent with such a hypothesis, oligonucleotide fingerprinting data show that the only detectable differences in the RNAs of FVP and FVA strains of SFFV are six oligonucleotides at the extreme 5' end of the RNA and two oligonucleotides at the 5' end of the envelope gene that are missing in SFFV_P RNA as well as the RNAs of F-MuLV and Friend MCF virus¹⁰. As the envelope message of RNA tumour viruses has been shown to contain sequences spliced from the 5' end of the genome^{20,21}, a signal sequence for the envelope gene of SFFV could be present in either of the areas of the SFFV genome which are different in SFFV_P and SFFV_A.

The differences in the processing of envelope glycoproteins encoded by SFFV_P and SFFV_A may be responsible for the different biological activities of these viruses. For example, the concentration of gp65^{env} on the cell surface could be important in determining the ability of proliferating cells to differentiate in the presence of various concentrations of erythropoietin. Alternatively, differences in biological activity and glycosylation could be independent consequences of a difference in the primary amino acid sequence of the envelope glycoproteins. Analysis of the biological effects of SFFV_P in conditions where glycoprotein processing is inhibited, sequencing of the envelope genes of molecularly cloned DNAs from SFFV_P and SFFV_A, and generation of mutants of molecularly cloned SFFV_P that biologically resemble SFFV_A should help to establish a relationship between the observed differences in glycoprotein processing and the different biological effects of these SFFV variants.

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Haemolysin contributes to virulence of extra-intestinal *E. coli* infections

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Escherichia coli is the predominant facultative microorganism isolated from the gastrointestinal tract of man and is the most common enteric organism causing extra-intestinal infections in man, particularly of the urinary tract, peritoneum and blood^{1,2}. It is likely that a consortium of virulence factors is responsible for the initiation and severity of extra-intestinal *E. coli* infections. Properties reported to be associated with the virulence of such infections include haemolysin production³, K1 antigen production^{4,5}, various O antigens⁵ and Fe sequestration⁶. For example, it has long been recognized that the ability to lyse erythrocytes is a phenotype more common to *E. coli* strains isolated from infections than those found in normal faeces^{3,7-9}. It is not clear whether the haemolysin *per se* is a virulence determinant. However, here we report that an isolated DNA sequence encoding haemolysin, added by recombinant DNA technology to avirulent non-haemolytic faecal isolates of *E. coli*, results in strains having enhanced virulence as measured in an experimental rat peritonitis model.

Several laboratories have demonstrated that the haemolysins commonly seen among animal faecal isolates of *E. coli* are encoded by transferable plasmids¹⁰⁻¹². There is evidence that these large plasmids confer virulence when transferred to avirulent strains of *E. coli*¹². However, it is not known whether the haemolysin itself or another unrecognized plasmid-mediated gene is responsible for this virulence phenomenon. Goebel and co-workers have demonstrated using DNA-RNA hybridization that plasmid-mediated haemolysin gene sequences are generally homologous with one another¹³. However, we have found that the haemolysin gene(s) of human *E. coli* clinical isolates most often resides on the bacterial chromosome¹⁴. Recently, chromosomal and plasmid-encoding haemolysin genes have been shown to share regions of DNA sequence homology, suggesting a common origin¹⁵.

We have previously shown¹⁴ by bacterial mating experiments that the haemolysin locus of the *E. coli* J96 isolate that causes human urinary tract infection resides on the bacterial chromosome. By cosmid cloning¹⁶, we have constructed a library of gene sequences of this organism in *E. coli* K-12. From a haemolytic *E. coli* K-12 clone we have identified and subcloned an 11.7-kilobase (kb) *SalI* restriction endonuclease DNA fragment encoding haemolysin and introduced this DNA fragment into the recombinant DNA vector pACYC184, resulting in a hybrid plasmid, pSF4000¹⁷. Using the ampicillin-resistance transposon Tn1 as a site-specific mutagen¹⁸, together with an *in vitro*-derived deletion, we have localized the haemolysin gene sequence to a 7.5-kb portion of pSF4000¹⁹.

Table 1 Effect of cloned haemolysin gene sequences on rat mortality in experimental intra-abdominal infections

Strain	Source	Haemolytic	Plasmid content	Rat deaths*/rats inoculated
J198	Human faeces	—	—	3/32
WAF 107	Transformation of J198	+	pSF4000	26/28
WAF 108	Transformation of J198	—	pSF4000::Tn1	0/20
WAF 111	Transformation of J198	+	pAN202-312	1/19

A single cryptic plasmid species is present in J198, which is colicin V-negative (ColV⁻).

* Number of deaths occurring within 7 days after receiving intraperitoneal injection of 10⁷ colony-forming units of each strain. Inocula were suspended in 10% barium sulphate (w/v)/50% sterile human faeces in brain heart infusion broth. Volume of inoculum in each case was 0.25 ml.

Figure 1 shows a restriction endonuclease fragment map of the haemolysin-encoding region of pSF4000.

The common laboratory strain of *E. coli* K-12 is considered to be an attenuated organism which cannot be converted to a virulent state by the acquisition of single virulence determinants^{4,5}. Therefore, appropriate testing of putative virulence properties should be done in the genetic background of 'wild' strains of *E. coli* represented by faecal isolates from healthy individuals. From our strain collection, a non-haemolytic 'wild' strain (J198) has proved useful because it can be transformed with plasmid DNA by the CaCl₂/cold shock technique of Lederberg and Cohen²⁰. This particular strain was also attractive for pathogenesis studies because we had found it to be avirulent for rats in an experimental intra-abdominal sepsis

may not be sufficient to engender virulence in a particular microorganism. Whether this reflects the structural differences between the 'human' and 'animal' haemolysins or the route by which the infecting microorganism is introduced into a susceptible host awaits further study. Recently, we have observed that another cloned haemolysin of animal origin confers slightly greater virulence than that of pAN202-312 in the rat model (R.A.W., E.P.D. and S.F., unpublished observations). These differences may lead to better understanding of the molecular basis of haemolysin action.

It was of interest to measure the level of virulence of our *in vitro*-derived pathogen (WAF 107) compared with the original clinical isolate J96 from which the haemolysin gene sequence was taken. It is apparent that the virulence of these two strains are similar quantitatively (Table 2). However, we are hesitant to conclude that acquisition of the haemolysin converts avirulent faecal strains into virulent organisms typical of those found in extra-intestinal infections for two reasons. First, we have noted that the course of infection differs for the two strains. Rats infected with J96 become symptomatic sooner and die earlier than those infected with WAF 107. In most cases, J96-infected rats died 12–24 h post-injection whereas most WAF 107-infected rats died between 24 and 36 h after infection. Second, there may be a significant difference in haemolysin gene dosage between clinical isolates and WAF 107 as our haemolysin gene sequence was cloned into a multi-copy plasmid vector. The significance of the dosage effect awaits further experimentation. Table 2 also shows representative LD₅₀ data for an *E. coli* K-12 prototroph harbouring the pSF4000 plasmid, which support the observations of other laboratories^{4,5} that the virulence of *E. coli* K-12 can be enhanced only slightly by the acquisition of any particular virulence property.

As *E. coli* infections of the urinary tract and other extra-intestinal sites are among the most common of human bacterial diseases, there is wide interest in understanding the precise mechanisms which contribute to the pathogenesis of these infections. Recombinant DNA technology represents a means of measuring the contribution of individual determinants of pathogenicity which are part of a polygenic virulence phenomenon. Although the precise mode of action of *E. coli* haemolysin in the pathogenesis of infection is still unclear, our data show that it has a significant role.

model²¹. This model mimics in two stages the disease process of peritonitis and multiple abscess formation seen in humans suffering intra-abdominal sepsis²¹. During the initial acute peritonitis phase in the rat model there is a high mortality rate associated with bacteraemic infections by *E. coli* or enterococci; here we have focused our attention on the role of putative *E. coli* virulence factors during this initial phase. The data presented in Table 1 illustrate a significant enhancement in rat mortality when pSF4000 is introduced into J198, which suggests that the haemolysin encoded by pSF4000 is responsible for the lethal behaviour of these strains in this animal model. In support of this, a Tn1 insertional mutant of pSF4000 which no longer confers the haemolysin phenotype does not confer virulence to J198 when present in the same genetic background. The particular Tn1 insertion used in the virulence studies (see Fig. 1) is in the region which encodes the structural gene for the haemolysin of pHly152 (ref. 22; R.A.W. and S.F., unpublished observations). The observation that the cloned haemolysin derivative pAN202-312 does not confer the same degree of virulence for the rat on J198 as pSF4000 is an interesting finding in view of their apparent sequence homology (Fig. 1). These data illustrate that similarity in phenotype and even close genetic homology

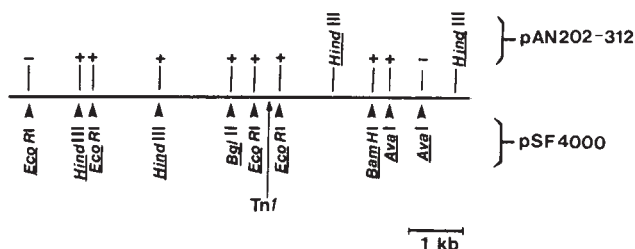


Fig. 1 Physical map of the haemolysin-encoding region of pSF4000. Restriction endonuclease cleavage sites for pSF4000 are shown below the bar. The pSF4000 plasmid was compared with pAN202-312 by restriction endonuclease fragment analysis. pAN202-312 is a recombinant plasmid (vector pACYC184) containing the haemolysin sequence derived from pHly152 (W. Goebel, personal communication); pHly152 was isolated from an *E. coli* strain found in mouse faeces¹³. The restriction sites common to both pSF4000 and pAN202-312 are designated by +. Restriction sites present in pSF4000 but absent from pAN202-312 are indicated by —. Two HindIII sites present in pAN202-312 but absent from pSF4000 are shown above the bar.

Table 2 LD₅₀ of selected strains in experimental rat intra-abdominal sepsis

Strain	Characteristics	LD ₅₀
J96	O4, ColV ⁺ , hly ⁺ , Hah ⁺	3.5 × 10 ⁵
J198	O22, ColV ⁻ , hly ⁻ , Hah ⁻	>10 ⁷
WAF 107	J198 transformed with pSF4000 DNA*	1.6 × 10 ⁵
W1485	K-12 prototroph, ColV ⁻ , hly ⁻ , Hah ⁻	>10 ⁷
WAF 109	W1485 transformed with pSF4000 DNA, ColV ⁻ , hly ⁺ , Hah ⁻	1 × 10 ⁷

The method of Reed and Muench²³ was used to determine the lethal dose for 50% of the population, LD₅₀. ColV⁺, colicin V production; hly⁺, haemolytic; Hah⁺, haemagglutination of human erythrocytes.

* pSF4000 encodes a haemolysin cloned from the J96 background.

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Physiological shear stresses enhance the Ca^{2+} permeability of human erythrocytes

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A membrane-bound Ca^{2+} pump, capable of extruding Ca^{2+} at a rate of $4\text{--}30 \times 10^{-3}$ mol Ca^{2+} per l of cells per h in optimal conditions¹, enables mammalian erythrocytes to maintain a large transmembrane Ca^{2+} concentration gradient (10^{-3}M outside compared with 10^{-6}M inside)². This rate is much greater than the passive Ca^{2+} influx, estimated³ to be $6\text{--}10 \times 10^{-6}$ mol per l of cells per h. It has been suggested that the apparently large excess capacity of the Ca^{2+} pump both gives the required Ca^{2+} sensitivity to a pump incapable of regulating its Ca^{2+} affinity⁴ and ensures against Ca^{2+} toxicity in certain red cell activities⁵. The results presented here, however, show that circulatory shear stresses enhance the Ca^{2+} permeability of the membrane, and suggest that greater demands are imposed on the Ca^{2+} pump *in vivo* than had been previously thought. We find that the passive permeability to Ca^{2+} is increased by up to an order of magnitude and the Ca^{2+} -stimulated ATPase activity is strongly enhanced when red cells are sheared at physiological rates in a rotational viscometer.

It has long been known that the shape and deformability of erythrocytes can influence blood viscosity⁶ and hence, in principle, blood flow in the circulation, but effects of fluid shear on cell membrane reactions have been demonstrated only recently^{7–9}. Here, examination of the effect of shear rate on Ca^{2+} uptake into ATP-depleted and ATP-rich human erythrocytes showed a shear-dependent increase in the uptake of $^{45}\text{Ca}^{2+}$ into the cells (Fig. 1), the uptake rate being constant for at least

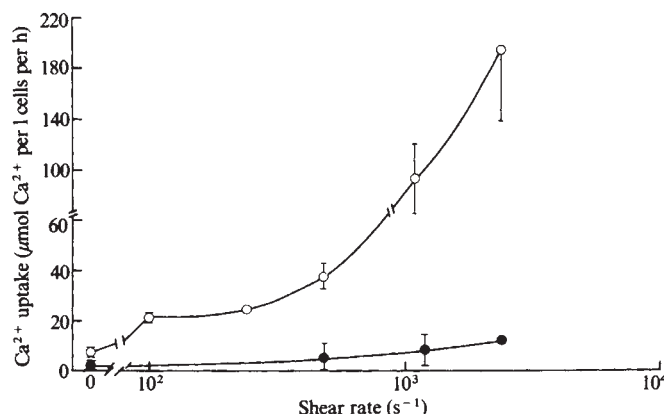


Fig. 1 Shear rate-dependent $^{45}\text{Ca}^{2+}$ uptake into human erythrocytes. Blood from a healthy volunteer was obtained by venipuncture with a 20-gauge needle in a 10-ml EDTA-vacutainer and tested the same day. Erythrocytes were separated from plasma by centrifugation at 3,000g for 10 min and washed twice in isotonic saline buffered with 20 mM sodium phosphate, pH 7.4. Packed cells were suspended at a haematocrit of ~10% in a solution containing 150 mM NaCl, 5 mM KCl, 15 mM Tris-HCl, 2 mM MgCl_2 , 5 mM iodoacetic acid, 5 mM adenosine and 0.1 mM EGTA at pH 7.4. Half of the sample was left on ice (ATP-rich) and the other half incubated at 37 °C for 3–4 h (ATP-depleted)¹⁶. Cells were then washed twice in the medium in which the $^{45}\text{Ca}^{2+}$ was to be measured: 150 mM NaCl, 5 mM KCl, 15 mM Tris-HCl, 2 mM MgCl_2 and 2 mM CaCl_2 . $^{45}\text{CaCl}_2$ was added (50–70 μl of a 0.5 mCi ml^{-1} solution) to 10 ml of the erythrocytes suspended at a haematocrit of 39.5–45%. The suspension was sheared in a variable-speed cone-plate Brookfield model LVT 'SynchroLectric' viscometer for 15–30 min at various shear rates at 37 °C. The uptake of Ca^{2+} into erythrocytes was assessed by a modification of the method reported by Lew and Ferreira⁴. Aliquots (100 μl) of the cell suspension before and after shearing were layered on 200 μl of Ficoll-Paque solution (Pharmacia) in a 400- μl centrifuge tube and spun for 2 min in a Beckman/Spinco 152 microfuge. The extracellular medium (top layer) and the Ficoll-Paque solution (middle layer) were removed by aspiration from the packed erythrocytes (bottom layer). A very slight red colour in the Ficoll-Paque solution was observed occasionally, indicating that minimal haemolysis had occurred at the shear rates tested. The walls of the centrifuge tube were wiped dry with a cotton swab and 100 μl of 10% perchloric acid were added to the tube. The tube was capped, vigorously shaken and the resulting precipitate packed by centrifugation for 1 min in the microfuge. Samples of 100 μl of supernatant were placed in 10 ml scintillation fluid (Amersham) and counted in a Beckman LS-3133P scintillation counter. Calcium uptake was expressed per l of cells per h based on the initial haematocrit values of the cell suspensions, as determined with a Clay-Adams micro-haematocrit centrifuge. The results shown are the means $\pm$ s.d. of four (ATP-depleted; ○) or three (ATP-rich; ●) determinations of cells from volunteer 1.

30 min. Figure 1 shows the increase in the rate of calcium accumulation by red cells depleted of their ATP following 15–30 min shear at various shear rates. Even at the lowest shear rate tested (120 s^{-1}) there was a significant increase ($P < 0.05$) in the Ca^{2+} taken up by the ATP-depleted erythrocytes compared with control cells which were only gently mixed. The increase in $^{45}\text{Ca}^{2+}$ associated with the cells was due to uptake and not binding to extracellular sites, as washing the cells in 10 times their volume of isotope-free buffer had no effect on the $^{45}\text{Ca}^{2+}$ uptake measured and ATP-rich erythrocytes showed a much lower $^{45}\text{Ca}^{2+}$ uptake (Fig. 1). The small $^{45}\text{Ca}^{2+}$ uptake seen in ATP-rich cells may reflect exchange with a pool of Ca^{2+} inaccessible to the pump rather than net uptake, as about 10–15% of the erythrocyte calcium is present in such a compartment^{10,11}. However, this fraction of $^{45}\text{Ca}^{2+}$ also seems to increase slightly with shear rate. We estimate this exchangeable pool of Ca^{2+} to be $4\text{--}5 \mu\text{mol Ca}^{2+}$ per l of cells, which agrees with previous reports¹¹.

The alternative explanation that the Ca^{2+} uptake into ATP-rich cells represents saturation of the Ca^{2+} efflux mechanism seems unlikely because the Ca^{2+} pump is capable of restoring Ca^{2+} to resting levels within a few minutes at 37 °C (ref. 12). Therefore, the most likely explanation for the greater $^{45}\text{Ca}^{2+}$ uptake in ATP-depleted than ATP-rich cells is that the ATP-dependent Ca^{2+} pump rapidly removes the Ca^{2+} taken up when sufficient ATP is present. This explanation is supported by experiments in which active Ca^{2+} extrusion from ATP-rich cells

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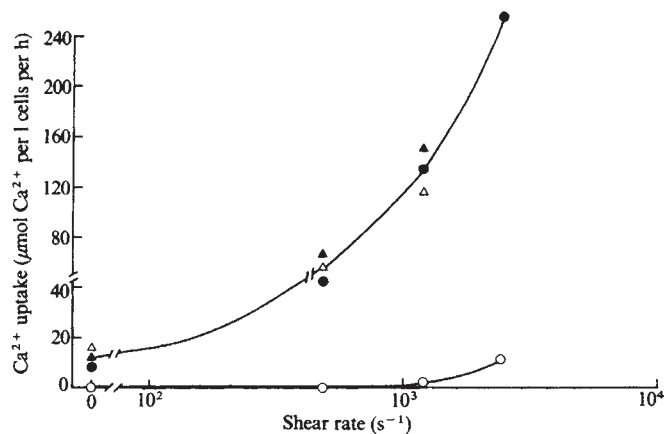


Fig. 2 Effect of La^{3+} on shear rate-dependent $^{45}\text{Ca}^{2+}$ uptake into human erythrocytes. The experiments were performed as described in Fig. 1 legend, except that 0.2 mM LaCl_3 was added to the erythrocyte suspension before shearing was begun. A single experiment from volunteer 2 is shown. ATP-rich cells in the absence of La^{3+} (○) and in the presence of 0.2 mM La^{3+} (●); ATP-depleted cells in the absence of La^{3+} (△) and in the presence of 0.2 mM La^{3+} (▲).

was blocked with La^{3+} (Fig. 2), which has previously been shown to abolish the active Ca^{2+} pump in resealed ghosts¹³ and intact cells¹⁴ without blocking the passive Ca^{2+} permeability¹⁵. In the presence of 0.2 mM lanthanum, $^{45}\text{Ca}^{2+}$ uptake showed the same shear dependence in both ATP-rich and depleted cells as found for ATP-depleted cells in the absence of La^{3+} . These results suggest that shear increases the Ca^{2+} permeability of the erythrocyte membrane through an effect on a La^{3+} -insensitive Ca^{2+} channel.

While the net accumulation of $^{45}\text{Ca}^{2+}$ into ATP-rich cells was only weakly shear dependent, Ca^{2+} -dependent ATPase activity in these cells increased strongly with increasing shear rate over the same range as that used in the Ca^{2+} uptake experiments (Fig. 3). The experiments were performed in ATP-rich cells treated with 5 mM iodoacetamide to limit glycolysis and the reincorporation of liberated P_i into ATP¹⁶. ($\text{Na}^+ + \text{K}^+$)ATPase and Mg^{2+} -ATPase activities were at most only slightly affected (Fig. 3). The Na^+ permeability may thus be much less shear sensitive, or iodoacetamide may have rapidly brought the Na^+ concentration in the cells to values saturating the Na^+ pump.

Circulating erythrocytes are subjected to periodic fluctuations in shear stress, conditions being most extreme in the micro-circulatory beds. These stresses induce continuous dramatic deformations of the cell membranes. The present results obtained with the physiological shear rate range⁶ of 50–

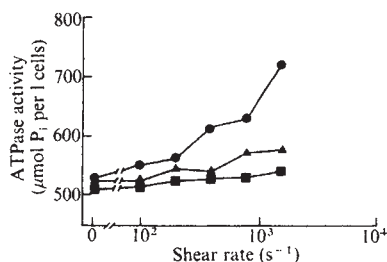


Fig. 3 Effect of shear rate on ATP turnover in ATP-rich human erythrocytes. ATPase activities were measured before and after shearing ATP-rich erythrocytes in the medium used in the $^{45}\text{Ca}^{2+}$ uptake experiments in Fig. 1, but which also contained 5 mM iodoacetamide. The contribution of ($\text{Ca}^{2+} + \text{Mg}^{2+}$)ATPase (●) was measured in the presence of 0.1 mM ouabain; Mg^{2+} -ATPase (■) was measured in the absence of CaCl_2 and the presence of 1 mM EGTA and 0.1 mM ouabain; ($\text{Na}^+ + \text{K}^+$)ATPase (▲) was measured in the absence of CaCl_2 . ($\text{Ca}^{2+} + \text{Mg}^{2+}$)ATPase activity was defined as the ouabain-insensitive activity determined in the presence of CaCl_2 minus the ATPase activity determined in the presence of 1 mM EGTA and no Ca^{2+} . Samples (200 μl) of erythrocyte suspensions were placed in 1.5-ml centrifuge tubes with 200 μl ice-cold 10% perchloric acid and centrifuged for 1 min in an Eppendorf 5412 centrifuge. Inorganic phosphate was determined in each 200 μl of the supernatant by the method of Fiske and Subba Row²². Results are from a single experiment from volunteer 1.

5,000 s^{-1} indicate that, coincident with such deformation, large increases in Ca^{2+} permeability occur. Hence, the level of internal Ca^{2+} in erythrocytes could show large transient increases in those parts of the circulation where shear stress is highest. Increased intracellular Ca^{2+} activity has been shown to increase the suspension viscosity of red cells without significantly affecting cell volume or unstressed shape¹⁷. Ca^{2+} -stimulated dephosphorylation of membrane substrates has also been correlated with the shape and shear resistance of erythrocyte membrane preparations¹⁸. The relationship between Ca^{2+} permeability, Ca^{2+} -dependent ATPase and pump activity, and intracellular biochemical events which are sensitive to Ca^{2+} levels may therefore be much more dynamic in the circulation than had previously been thought. Viewed in this light, even the twofold deficiencies in Ca^{2+} pump activity seen in red cells from cystic fibrosis patients¹⁹ or in sickle cells^{20,21} could acquire greater significance than has hitherto been afforded them.

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Alisphenoid equivalents in placentals, marsupials, monotremes and fossils

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The interpretation of the wall of the brain case between orbit and ear has important implications for mammalian phylogeny. The fossil mammalian groups Morganucodonts, Triconodonts and multituberculates possess a reduced alisphenoid and a large anterior process of the petrosal. In this they resemble monotremes. By contrast 'therians' (marsupials, placentals and their putative ancestors) have a large alisphenoid and no anterior process. Palaeontologists reject forms with a large anterior process from a position close to the ancestry of therians, though uncertain of the timing of the divergence^{1,2}. The development of the brain case of monotremes is crucial here. The view that their anterior process is a laminar outgrowth of the petrosal³ has been widely accepted. Recent work has shown that this is not true in echidnas^{4,5} and here I confirm the suggestion⁶ that it is not true in platypus. The ossification pattern of living mammals indicates the possibility of close affinity between 'therian' and 'non-therian' mammals.

The area of interest develops in the lateral wall of the cavum epiptericum, the cartilaginous ala temporalis in front and the cartilaginous otic capsule (future petrosal) behind. In all mammals a fibrous speno-obturator membrane⁷ lies between these, extending up to the edge of the chondrocranium lateral to the ophthalmic nerve. Terminology of this area has varied. Here I use 'processus ascendens' for the part of the ala between ophthalmic and maxillary nerves; 'lamina ascendens' for the part of the ala between maxillary and mandibular nerves. The English word 'process' signifies an adult osteological feature and 'lamina' is confined to development. I propose here that the more recent term 'lamina obturans'⁴⁻⁶ used for membrane bone developing in the speno-obturator membrane of monotremes may usefully be extended to the very similar field of membrane bone here in therians. This membrane bone is very variable in its development and fate in Recent mammals and may well be the key to the evolution of this region in advanced therapsids.

I present here two stages from a developmental series of serially-sectioned platypus nestlings. The younger specimen has a small lamina obturans: a single sheet of membrane bone entirely separate from the otic capsule and ala temporalis (Fig. 1a). The older specimen, used by Watson³, was the only one available to him having a lamina suitable for histological study. He described it as a forward outgrowth of the petrosal, founding the prevalent morphological view. In this specimen the area of synostosis between the lamina obturans and endochondral bone in the otic capsule is very small (Fig. 1b); in the younger specimen the otic capsule shows no sign of endochondral ossification here. It is clear from these and other specimens that the platypus lamina obturans is an independent centre of intramembranous ossification within the speno-obturator

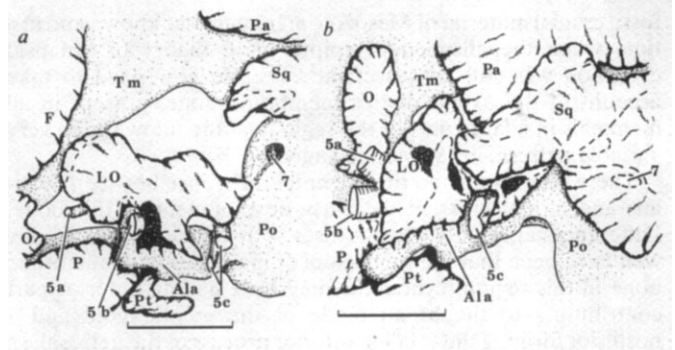


Fig. 2 Temporal regions of *a*, *Didelphis virginiana*, 24 mm crown-rump (CR), lateral aspect; *b*, *Erinaceus europaeus*, 14.5 mm head length (HL), lateral aspect. Same scale as in Fig. 1. There is an extensive area of membrane bone with similarity to the monotreme lamina obturans in each, but there is little endochondral ossification in the ala temporalis. The dark areas in each lamina indicate schematically where, in younger specimens (14 mm CR opossum, 13 mm HL hedgehog), the earliest ossification in membrane bone was found with no evidence of ossification in adjacent cartilage. The great variability of the ala temporalis in mammals is illustrated by the presence in the opossum of a cartilaginous processus ascendens between 5a and 5b, while the hedgehog lacks this but has a lamina ascendens between 5b and 5c.

membrane, analogous to the multiple centres described in echidnas⁵ (Fig. 1c). Watson³ lacked younger platypus having this bone and overlooked the possibility of its independent origin.

The structure of this region in monotremes may be summarized as follows: in platypus the small ala temporalis ossifies quite early, with a small perichondrial extension into the speno-obturator membrane ('lamina ascendens'⁵). A single lamina obturans appears which fuses first with the petrosal and later with orbitosphenoid, squamosal, palatine and 'lamina ascendens'⁵. Other workers^{4,5} have shown that the echidna lamina obturans appears late as several centres; my specimens show here early signs of ossification consistent with these reports, but lack later juvenile stages. The ala temporalis is smaller than that of the platypus but ossifies early and produces a larger perichondrial extension ('lamina ascendens'⁵) into the speno-obturator membrane, as do the palatine and the anterior margin of the petrosal. In echidnas, closure of the suture between lamina obturans and petrosal may be later than with adjacent elements⁵. It is still visible, though unlabelled, in Watson's³ figures of echidna (G. T. MacIntyre, personal communication).

All recent therians have a speno-obturator membrane, in which membrane bone appears earlier than in monotremes, often before endochondral ossification in the ala temporalis, unlike the monotreme 'lamina ascendens'⁵. It appears close to the ala and extends back to suture with the squamosal and tegmen tympani. It is very variable in shape and number of centres. The opossum (Fig. 2a) has a pattern similar to the platypus; the hedgehog (Fig. 2b) has three centres. This membrane bone is histologically and anatomically similar to the lamina obturans of monotremes and forms most of the blade of the alisphenoid of therians, the ala temporalis forming a usually smaller portion from root to nerve exits.

Previous analysis⁸⁻¹⁰ holds that the therian alisphenoid, while histologically a complex of membrane and cartilage bone, should be treated morphologically as a derivative of the epipterygoid of primitive tetrapods, the membrane bone being merely a large perichondrial extension of the processus ascendens of the epipterygoid which has been modified and reduced to the ala temporalis. This view is simple and very effective, but note that it was founded before information on the true development of monotremes was available, before excellent

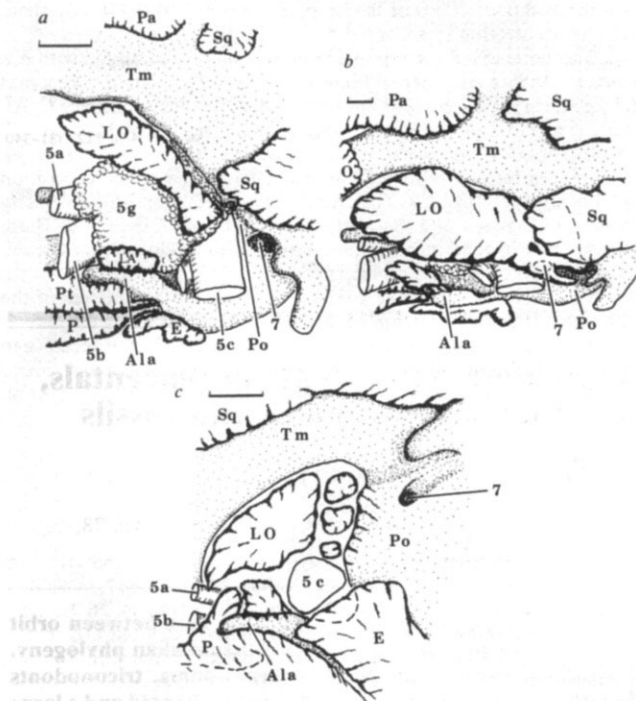


Fig. 1 Temporal regions of *a*, *Ornithorhynchus anatinus*, 168 mm snout-tail (ST), lateral aspect; *b*, *O. anatinus*, 250 mm ST, lateral aspect; *c*, *Tachyglossus aculeatus*, 174 mm ST, ventral aspect. Scale bar, 1 mm. Cartilage is shown stippled, bone with edges scalloped. *a* Shows a large lamina obturans (LO) barely touching the petrosal (Po). *b* Has the lamina synostosed with the petrosal in the area overlapped by the squamosal (Sq). *c* Is drawn from the oldest available specimen: the temporal wing of the palatine and 'lamina ascendens'⁵ (LA) were ossified but the position of other ossifications in the membrane and petrosal are based on refs 4, 5. Ala, ala temporalis; E, ectopterygoid; P, palatine; Pt, pterygoid; Tm, taenia marginalis. 5a,b,c,g, Ophthalmic, maxillary and mandibular nerves and trigeminal ganglion, respectively. 7, Foramen of facial nerve.

fossil cranial material of Mesozoic mammals was known and at a time when the alisphenoid-epipterygoid theory of synapsid evolution was still controversial^{3,11,12}. We now need to take account of the extent of this membrane bone, present in all mammals in a large part of the region, and to allow for its very variable pattern, in taxonomic analysis.

There seems to be no fundamental difference between therians and monotremes in the early development of this bone. Differences arise only when it fuses with its neighbours. It may well have been that the precursors of mammals had membrane bone in this region: cynodonts may have had an anterior part contributing to the broad blade of the epipterygoid and a posterior forming the smaller anterior process of the petrosal. In other groups the proportions may have varied: in triconodonts the anterior process is larger and the epipterygoid blade smaller, while in multituberculates the membrane-bone area may have contributed extensively to the petrosal to the exclusion of the diminutive alisphenoid (epipterygoid). In view of the variable pattern found in living forms and the difficulties of establishing detailed osteological homologies in fossils, it must surely be dangerous to erect two groups¹, however informally, based on

the adult pattern of this region. Development shows that there is no 'monotone pattern' and therefore fossils may not be categorized as sharing it.

Excellent skull material of morganucodonts¹, triconodonts² and multituberculates^{13,14} is now being studied. It must be appreciated that the blade of the alisphenoid and the anterior process of the petrosal are developmentally very similar and may be morphologically almost equivalent. If Mesozoic, like Recent, mammals formed membrane bone in the area of the lamina obturans, it follows that any form in the fossil record with an expanded epipterygoid, an anterior process of the petrosal, or both, could, by a simple change in the affinity of synostosis during development¹⁵, come to possess therian anatomy. Therefore the recognition of the precursor form of therians must rest on other features. The structure of the wall of the cavum epiptericum itself in morganucodonts, triconodonts, multituberculates, cynodonts or even monotremes does not exclude close affinity to therians.

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Corrigenda

A correction to the letter '²¹⁰Pb in surface air at Enewetak and the Asian dust flux to the Pacific' by Karl K. Turekian & J. Kirk Cochran, *Nature* **292**, 522–524.

We have just discovered that the filters sent to us for ²¹⁰Pb assay and reported in our recent paper¹ were one-quarter aliquots of the total filter and that the values for volumes transmitted to us subsequently were for the whole samples. This means that Table 1 in our paper must be revised to increase the ²¹⁰Pb concentration in Enewetak air by a factor of 4: the correct version is shown below.

Table 1 ²¹⁰Pb and ²¹⁰Po in air filter samples from Enewetak, April–August 1979

Date of sampling	²¹⁰ Pb (d.p.m. 10 ⁻³ m ⁻³)	²¹⁰ Po/ ²¹⁰ Pb activity ratio
18 April	15.3 ± 2.1	—
26 April	12.1 ± 1.0	−0.02 ± 0.08
3 May	10.4 ± 0.76	−0.02 ± 0.03
10 May	12.2 ± 1.0	−0.02 ± 0.03
15 May	7.96 ± 0.72	−0.08 ± 0.03
20 June	5.96 ± 0.20	0.14 ± 0.11
8 July	8.00 ± 0.32	0.02 ± 0.28
16 July	3.17 ± 0.14	−0.26 ± 0.04
26 July	3.11 ± 0.076	0.17 ± 0.07
4 August	4.04 ± 0.16	0.05 ± 0.07
Tower blanks (filter carried up the sampling tower in exposed conditions)		
10 May	0.424 ± 0.108	−0.09 ± 0.10
26 July	0.180 ± 0.032	−0.05 ± 0.18

Sampling date represents the date corresponding to the midpoint of the collection period. Errors are 1σ counting errors.

This correction changes several relations that we discussed.

(1) Both the ordinate values for Fig. 1 and the abscissa values in Fig. 2 should be increased by a factor of 4.

(2) The corrected Enewetak filter results (present Table 1) compare more favourably with previous observations made at Hawaii². Between 15 May and 20 June 1979 at Enewetak, the concentration of ²¹⁰Pb was 6–8 d.p.m. 10⁻³ m⁻³ and for a similar period (30 May–22 June 1971) at Hawaii, the values below the trade wind inversion were 3.4–19.4 d.p.m. 10⁻³ m⁻³ with a mean of 9.3 d.p.m. 10⁻³ m⁻³.

(3) The regression equation for the calculation of the mean annual concentration of ²¹⁰Pb in air becomes

$$C \text{ [d.p.m. } ^{210}\text{Pb } 10^{-3} \text{ m}^{-3}] = 18.8 - 85.6 \times F \text{ [d.p.m. } ^{210}\text{Pb cm}^{-2} \text{ yr}^{-1}]$$

For a mean flux of ²¹⁰Pb of 0.15 d.p.m. cm⁻² yr⁻¹ this yields a mean annual concentration in air of 6 d.p.m. ²¹⁰Pb 10⁻³ m⁻³.

(4) The slope (S) of the regression equation correlating F_{Al} with F_{Pb} becomes 9.5 μg Al per d.p.m. ²¹⁰Pb. F_{Al} then becomes 0.63 μg Al cm⁻² yr⁻¹ corresponding to a dust flux (assuming 6.5% Al) of 9.6 ± 5 μg cm⁻² yr⁻¹, a closer value to that reported by Duce *et al.*³.

(5) In Table 2 of our paper¹ the increase by a factor of 4 of the mean ²¹⁰Pb concentrations reported there decreases the 'total deposition velocities' by a factor of 4. Thus 'dry' season total deposition velocity becomes 0.25 cm s⁻¹ and the 'wet' season becomes 1.0 cm s⁻¹. These values are now lower than our estimated deposition velocities for Hawaii (~3 cm s⁻¹) and Bermuda (~4 cm s⁻¹).

(6) The dust flux calculated for Hawaii using the parameters of the Enewetak ²¹⁰Pb–Al regression equation now becomes 93 μg cm⁻² yr⁻¹. This is easily in the range of accumulation rates of clay on the deep ocean floor. The conclusion regarding the dominance of the Asian dust flux to the sedimentation in the Pacific remains intact as does the suggestion that post depositional homogenization has occurred.

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In the letter 'Is the similarity of monozygotic twins due to genetic factors alone' by K. Gärtner and E. Baunack, *Nature* **292**, 646–647, in Table 1 mean ± s.d. of the MZ should be 26.41 ± 2.58 instead of 26.2 ± 2.66, and in Tables 1 and 2 the s_b^2 values of 51- and 61-day old DZ should be −1.18 and −2.49 instead of the printed positive values. In Table 2, the calculated F values of the s_b^2 of MZ and DZ are statistically insensitive; therefore the F and P values of s_b^2 should be disregarded.

The title of the letter by Doyle R. Watts and Andrew M. Bramall in *Nature* **293**, 638–641 should read 'Palaeomagnetic evidence for a displaced terrain in Western Antarctica'.

In the letter 'SB: a new HLA-linked human histocompatibility gene defined using HLA-mutant cell lines', *Nature* **293**, 747–749, the present address of Fritz H. Bach should read 'Immunobiology Research Center, University of Minnesota, Minneapolis, Minnesota 55455, USA'.

In the review article 'A new era in mammalian gene mapping: somatic cell genetics and recombinant DNA methodologies' by F. H. Ruddle, *Nature* **294**, 115–120, on p. 119 'α-globin' should read 'β-globin' throughout. In Table 1, the chromosome positions for the mouse α-globin pseudogenes are sited incorrectly: pseudogene ψ3 maps to chromosome 15 and pseudogene ψ4 to 17.

MATTERS ARISING

Presence and physiological role of presynaptic inhibitory α_2 -adrenoreceptors in guinea pig atria

It was concluded by Angus and Korner¹ that "presynaptic α -adrenoreceptor modulation by synaptically released noradrenaline plays no part in cardiac sympathetic transmission".

Evaluation of their results and experimental methodology suggests that this criticism may be premature because there are important methodological differences between this study and several others which showed evidence for presynaptic α -adrenoreceptor modulation of cardiac neurotransmission. As the consensus of opinion supports a physiological role for presynaptic α -adrenoreceptors in the heart, it is useful to identify and examine these experimental differences in more detail.

(1) The study was carried out with guinea pig right atria in Krebs' solution which contains 1.2 mM Mg^{2+} . In these experimental conditions the resting rate¹ is only 110 beats per min while in most articles dealing with spontaneously beating guinea pig atria and using Locke's solution, which does not contain Mg^{2+} , the resting rate²⁻⁵ is between 200 and 230 beats per min.

Angus and Korner state that "phenolamine 0.3–10 μ M had no significant effect on the resting atrial rate or the dose-response curve to noradrenaline"¹. The fact that their resting rate was already very low¹ may explain their findings.

However, the authors¹, without admitting their different resting atrial rate, presented these results as conflicting with findings by Langer *et al.*⁶ of a negative chronotropic effect and attenuated chronotropic responses to exogenous noradrenaline in the presence of 31 μ M phenolamine. However, the results of Langer *et al.*⁶ were obtained with Locke's solution in conditions in which the resting atrial rate⁶ was 210 beats per min. When repeating their experiments with Locke's solution Angus and Korner found that, as reported previously⁶, even 10 μ M phenolamine produced a significant negative chronotropic effect in guinea pig atria (J. A. Angus, personal communication). Angus and Korner did not determine whether 31 μ M phenolamine antagonized responses of atria to exogenous noradrenaline as reported by Langer *et al.*⁶.

(2) Instead of accelerans nerve stimulation⁶ the experiment by Angus and Korner used field stimulation¹; clearly, the latter is a less physiological stimulus than nerve stimulation, and this difference in methodology may be one of the reasons

for their negative results. In support of the results of Langer *et al.*⁶ it was recently reported that both phenolamine and yohimbine produce a significant enhancement of the inotropic responses to sympathetic stimulation in guinea pig left atria⁷.

(3) Potentiation of the chronotropic responses to accelerans nerve stimulation at 0.5 Hz was reported in the presence of 0.1 μ M phenolamine⁶. Using one or four pulses of field stimulation applied during the refractory period, exposure to 10 μ M phenolamine failed to potentiate the chronotropic responses to field stimulation¹. Recent experiments in which field stimulation was applied in guinea pig atria using four pulses at 2 Hz showed that exposure to 3 μ M phenolamine did not significantly affect the overflow of ³H-noradrenaline⁸, thus confirming the observation of Angus and Korner¹. However, when field stimulation was applied at 2 Hz using a total of 16 pulses, exposure to 3 μ M phenolamine enhanced the overflow of the labelled transmitter as well as the peak chronotropic responses to stimulation⁸. Obviously, the negative feedback regulation of noradrenaline release mediated by presynaptic α_2 -adrenoreceptors depends on the frequency and duration of neuronal activity. Failure to observe potentiation of the positive chronotropic responses to field stimulation in the report by Angus and Korner¹ is clearly related to the fact that they used too short a period of stimulation. These results⁸ confirm the findings of Langer *et al.*⁶ obtained with accelerans nerve stimulation.

It is premature to reach definitive conclusions, as did Angus and Korner, about the absence of feedback modulation of noradrenaline release based on negative results originating from inadequate experimental conditions which are restricted to very brief periods of stimulation. If these authors had explored a wide variety of frequencies of stimulation using different durations of the period of stimulation, their conclusions would have been quite the opposite, as clearly demonstrated by Story *et al.*⁸.

(4) Although the presence of presynaptic inhibitory α_2 -adrenoreceptors in guinea pig atria is questioned by Angus and Korner¹, they have shown subsequently that clonidine, an α_2 -adrenoreceptor agonist, decreases the responses to field stimulation in this tissue⁹. In addition, clonidine reduces the inotropic response to sympathetic nerve stimulation in guinea pig left atria and this effect is antagonized by 5 μ M phenolamine or 0.1 μ M yohimbine (ref. 7). These results clearly support the view that presynaptic inhibitory α_2 -adrenoreceptors are present in noradrenergic nerve endings of the peripheral nervous

system¹⁰⁻¹². These release-modulating presynaptic α_2 -adrenoreceptors are definitely of pharmacological relevance in both *in vitro* and *in vivo* conditions^{13,14}. The possible physiological role of presynaptic α_2 -adrenoreceptors in noradrenergic neurotransmission is supported by the findings that α_2 -adrenoreceptor blocking agents enhance both the electrically evoked release of noradrenaline and the end-organ responses to sympathetic nerve stimulation¹⁰⁻¹² provided suitable experimental conditions are used.

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ANGUS AND KORNER REPLY—In our paper¹ we tried to establish whether or not neurally released noradrenaline could activate an auto-inhibitory feedback on the sympathetic nerve terminals of the guinea pig right atrium and concluded that it did not. There were differences between our methodology and those of Langer and co-workers and we are grateful for the opportunity of discussing these.

(1) Bath solution. We used a standard bicarbonate-buffered solution for heart muscle²⁻⁴ of pH 7.58 ± 0.01 ($n = 5$) bubbled with 95% O₂, 5% CO₂, compared with Langer and co-authors⁵⁻⁷ who routinely use Mg^{2+} -free Locke's solution bubbled with 100% O₂. We found that this Locke's solution has a pH of 8.19 ± 0.06 ($n = 4$), a similar finding to Trendelenburg⁸ and well outside the normal physiological range. The higher resting rate in Locke's solution of 180–210 beats per min compared with our rate of 110–150 beats per min are accounted for by the well known effect of pH on resting atrial rate^{9,10} rather than the lack of Mg^{2+} which, parenthetically, is present in guinea pig

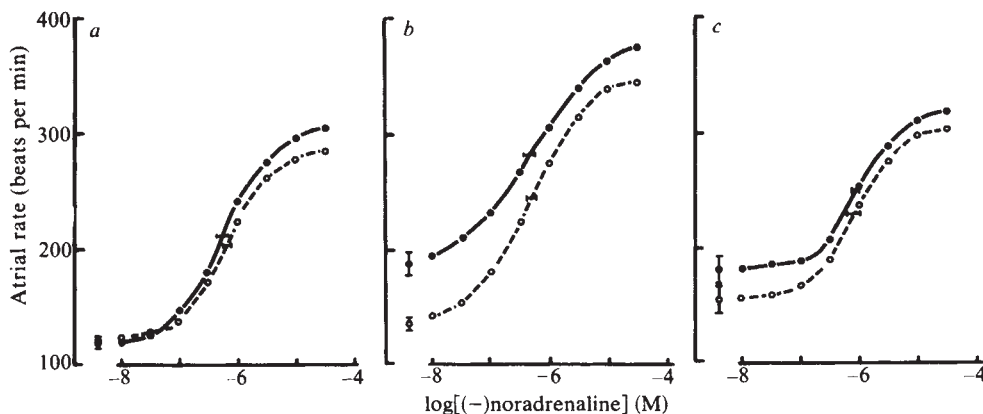


Fig. 1 Effect of 10 μ M phentolamine (30 min contact) on resting atrial rate and on chronotropic responses to exogenous (—)noradrenaline in isolated guinea pig right atria in three different bath conditions. *a*, Krebs' solution, pH 7.58, temperature 37 °C ($n=4$): control, first curve; phentolamine, fifth curve (data from ref. 1). *b*, Krebs' solution, pH 7.58, temperature 38.4 °C ($n=6$): control, first curve; phentolamine, second curve. *c*, Locke's solution, pH 8.19, temperature 37 °C ($n=4$): control, first curve; phentolamine, second curve. Solid symbols = control; open symbols = phentolamine. Means $\pm$ s.e.m. at the left of each graph are the resting atrial rates before the start of the noradrenaline curve.

plasma (0.86 ± 0.08 mM, $n=4$). As pointed out by Blinks and Koch-Weser¹¹, "the solution that produces the most vigorous contractions is not necessarily the most physiological one... The most physiological solution is one that most closely resembles the normal extracellular fluid..."

(2) Phentolamine. We agree that the lower resting rates in our preparations¹ may have masked the negative chronotropic effect of high phentolamine concentrations (10 μ M). By raising the temperature of the bath from 37 °C to 38.4 °C (body temperature in conscious guinea pigs) the resting rates were now 186 ± 29 ($n=6$), significantly higher than at 37 °C. In these conditions, as with Locke's solution at 37 °C, 10 μ M phentolamine significantly lowered the resting rate (Fig. 1). However, the sensitivity (threshold and ED_{50} value) and range (change in rate) of the exogenous noradrenaline concentration-response curves were not significantly altered by phentolamine (negative log molar ED_{50} values: Krebs', 38.4 °C ($n=6$), control = 6.33 ± 0.11 , phentolamine 6.31 ± 0.09 ; Locke's 37 °C ($n=4$), control = 6.03 ± 0.03 , phentolamine 6.08 ± 0.09). Therefore, we cannot conclude that 10 μ M phentolamine antagonizes the atrial responses to noradrenaline, a suggestion used by Langer and co-authors to explain why phentolamine (3.1 μ M) failed to potentiate atrial responses to nerve stimulation on two occasions^{5,6} despite a large but variable increase in 3 H-noradrenaline release. It is interesting that Langer⁵ has observed an increase in the rate response after 0.31 μ M phentolamine with nerve stimulation at 0.5 Hz for 10 s, but that in the same study⁵ the supporting evidence of increased noradrenaline release was made at nerve stimulation of 4 Hz for 60 s, a stimulus that produces near maximum rate response⁶. Higher concentrations of phentolamine (31 μ M) appeared to depress the resting atrial rate and to shift the exogenous noradrenaline concentration-response curve to the right⁵. The relevance of these data is not clear, as Langer and colleagues⁵ presented no results on 3 H-noradrenaline release or rate response at this concentration.

(3) Accelerans nerve stimulation. The advantage of field stimulation is that all nerve varicosities are depolarized synchronously by each field pulse¹². With nerve stimulation there is some evidence^{13,14} that action potentials may not always invade all the terminal varicosities. Stjärne¹⁵ points out that drugs may potentiate noradrenaline release by recruitment of varicosities. Therefore, to test the hypothesis of auto-inhibitory feedback, it is logical to eliminate varicosity recruitment by using field stimulation, a technique used by others in examining noradrenaline release in atria⁴.

Therefore, we believe that the methodological points raised by Langer do not invalidate our conclusion that physiologically released noradrenaline does not activate auto-inhibitory receptors in the conditions of our assay. If the widely championed view that presynaptic α -adrenoreceptors are important in sympathetic transmission in physiological conditions, we assert that phentolamine should have raised responses after four pulses. After all, a "pulse-to-pulse modulation of noradrenaline release" was proposed by Rand and colleagues¹⁶ and has often been quoted by Langer¹⁷.

Recently, as Langer points out, Story and colleagues have confirmed our results that the tachycardia resulting from four field pulses that give up to 50% maximum response is unaffected by phentolamine. However, an increase in response was observed in the presence of phentolamine after 16 field pulses, which cause near maximal tissue response. In our experiments, field pulses were delivered only in the atrial refractory period to avoid arrhythmia, in marked contrast to the random delivery in experiments by Story *et al.*¹⁸. They have now described conditions in which the feedback loop seems to operate even for four field pulses provided the frequency is ≤ 1 Hz. If the conditions for the operation of the feedback loop are so critical, it is difficult to imagine how a nerve varicosity can be involved in feedback control in normal conditions when not every impulse from nerve trunk stimulation invades each varicosity.

We too have found that clonidine inhibits the sympathetically mediated

tachycardia and that this action is antagonized by low concentrations of phentolamine (0.1 μ M)¹⁹. Therefore, we agree that there is an important pharmacological target on cardiac sympathetic nerve terminals which is probably related to a presynaptic α -adrenoreceptor and where some imidazoles show high affinity.

Finally, our paper has, of course, not examined all conditions of stimulation because we attempted to examine the role of presynaptic α -adrenoreceptors in conditions producing a moderate tissue response. Clearly, our paper and that of Story *et al.* highlight the need for caution in assuming a general operation of auto-inhibitory feedback in cardiac sympathetic transmission.

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Satellite-sensed turbulent ocean structure

RECENTLY Gower *et al.*¹ have reported interesting results concerning the concentration of phytoplankton as measured by the Landsat multispectral scanner on 19 June 1976 in a region south of Iceland. The observed concentration fluctuation spectra is found to follow approximately a $k^{-2.92}$ law, close to the k^{-3} law proposed by Charney² for the energy spectrum of quasi-geostrophic flows. They conclude that this result is consistent with the hypothesis that phytoplankton behaves like a passive scalar convected by ocean currents.

The quasi-geostrophic theory of Charney² predicts k^{-3} laws for both horizontal kinetic energy and temperature variance, however, temperature is not a passive scalar, but the vertical derivative of the horizontal stream function. It is the constraints of total (kinetic plus available potential) energy conservation and potential enstrophy conservation

$$\frac{d}{dt} \langle \psi_x^2 + \psi_y^2 + \psi_z^2 \rangle = 0$$

$$\frac{d}{dt} \langle \psi_{xx}^2 + \psi_{yy}^2 + \psi_{zz}^2 \rangle = 0$$

together with the assumption of statistical isotropy, which yields for any of the derivatives ψ_x, ψ_y, ψ_z a power spectrum following a k^{-3} law in the potential enstrophy cascading range.

Now, if we consider a passive scalar convected by the quasi-geostrophic motion, as we may expect phytoplankton is, the situation must be quite different. For simplicity we neglect baroclinic effects: for pure two-dimensional turbulence, it can be shown³ using either phenomenological arguments or statistical closures, that the spectrum of a passive scalar is proportional to the spectrum of the quantity which cascades. Therefore in the enstrophy cascading inertial range, the spectrum of a passive scalar should follow a k^{-1} law, like the enstrophy (and not the energy) spectrum. There seems to be no reason why this result could not be extended to quasi-geostrophic turbulence which follows a similar phenomenology. A k^{-3} energy spectrum for the ocean currents would then imply a k^{-1} law for the passive scalar; conversely, a k^{-3} spectrum for the passive scalar variance of the type reported by Gower *et al.*¹ would imply a much steeper slope (k^{-5}) for the current kinetic energy.

Such steep slopes have been found in fact in numerical simulations of forced two-dimensional turbulence⁴; the consequent discrepancy with the Kolmo-

gorov-Kraichnan-Leith theory⁵ which predicts a k^{-3} law is still an open question. For instance, the presence of intermittency effects would yield a steepening of the energy spectrum⁴; but still, neither kinetic energy spectra nor temperature variance spectra steeper than k^{-3} have yet been observed experimentally.

The quasi-geostrophic theory of Charney² neglects the influence of upper and lower boundaries; it is therefore likely to be valid in the deep ocean rather than near the surface. Near the surface the dynamics is likely to involve the occurrence of frontal systems, and one should refer to the quasi-geostrophic theory of Blumen⁶ rather than to that of Charney². In Blumen's⁶ theory potential vorticity is assumed to vanish

$$\psi_{xx} + \psi_{yy} + \psi_{zz} = 0$$

inside the fluid, and the two invariants are the total energy of the fluid, and the available potential energy at the surface, which both reduce to integrals taken over the surface only:

$$\frac{d}{dt} \langle \psi_z^2 \rangle = 0$$

$$\frac{d}{dt} \langle \psi_{zz}^2 \rangle = 0$$

The quantity which cascades to smaller scales is the available potential energy at the surface, which therefore exhibits a $k^{-5/3}$ law characteristic of frontal systems. If we follow this theory, which looks more appropriate than the first one in the case of surface dynamics, we end up with a $k^{-5/3}$ law of the passive scalar variance. This is again quite far from what has been observed for phytoplankton, although again the presence of intermittency could yield a steeper slope.

It seems, therefore, difficult to infer anything conclusive from the measurements reported by Gower *et al.*¹ concerning a possible behaviour of phytoplankton as a passive scalar. Also, some caution

should be observed regarding regression estimates for spectral slopes. The concept of an inertial range is only asymptotically valid, and the data used by Gower *et al.*¹ may be close to the internal radius of deformation to yield any sensible approximation to the actual behaviour of the inertial range. Apparently a regression calculated on the range 1–10 km would yield much lower values of the slope.

A secondary point concerns the assumptions in Gower *et al.*¹ that radiance upwelling is a linear function of phytoplankton concentration. The only assumption needed to investigate the passive scalar character of phytoplankton is that radiance upwelling is a function of phytoplankton concentration only, because any function of a convected quantity is a convected quantity, exhibiting therefore the same spectral behaviour.

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GOWER ET AL. REPLY—In our recent paper¹, the -2.92 slope found for the spectrum of near-surface phytoplankton variance is not consistent with theories of a passive scalar in a quasi-geostrophic turbulent fluid. The reasoning is based on a subsequent paper by Lesieur *et al.*²

However, we do not feel that a further search for physical explanations for our spectrum is warranted: phytoplankton are not always a conserved passive scalar. Rather, as microscopic plants in the sea, they grow, at times at rates capable of doubling their weight each day; and they die or are eaten, often at rates comparable with their growth rates. These time scales are short compared with typical time scales for quasi-geostrophic eddies (10 days). Even in the absence of turbulent motion, biological processes can result in similar negative power law spectra³. Still it is feasible that areas of abundant phytoplankton may correlate with fluctuations of a dynamic variable, such as upwards

Matters Arising

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velocity or temperature, in which case the spectra of temperature and phytoplankton variance might be similar.

The point that all convected quantities in a quasi-geostrophic turbulent field will have the same variance spectrum even if they are related in a non-linear manner, if it is correct, increases our confidence that our published spectra are an accurate representation of the near surface phytoplankton variability. We can still envision that constraints of calculating a spectrum, such as the apparent diffusion caused by the finite size of picture elements and the necessity to average over picture elements, might cause convected quantities related in a non-linear manner to have different calculated spectra.

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Problem of light piping in immunofluorescence studies

In providing evidence from indirect immunofluorescence for the presence of myosin in the stereocilia of cochlear hair cells, Macartney *et al.*¹ present a figure in which fluorescent light, in the pattern of stereocilia, emanates from the apical surface of hair cells. As the arrangement of the actin filaments which form the core of the stereocilium places structural constraints on the location of significant amounts of other proteins, if myosin is present in the stereocilia, it would have to form either the inter-filament cross-bridge or be located in the space between the core and the membrane, both of which seem unlikely. Therefore the results of Macartney *et al.* were puzzling. This led me to consider the possibility that they were an artefact of light piping. I have subsequently learned that Macartney *et al.* (personal communication) have taken transverse sections of the organ of Corti, showing fluorescence along the lengths of the stereocilia, thus making light piping a less likely explanation of their results. Nevertheless, for the sake of future studies on the localization of immunofluorescence in hair cells we now explain the problem of light piping so that precautions can be taken to exclude it as a source of spurious results.

Light piping was first observed with respect to hair cells in the last century, and

has been described more recently by Kohlloffel³. Hair cells, because they have a higher refractive index than the surrounding extracellular medium, behave as optical waveguides (the 'fibre optic' effect); light entering these cells obliquely Kohlloffel's treatment of this phenomenon³ was restricted to the optical phenomenon³ was restricted to the optical properties of the hair-cell body (which is roughly cylindrical in shape), but other observations suggest that the stereocilia, emerging from the apical surface of the cell, can enhance this effect.

It would be convenient if one could make a detailed description of the exact optical properties of the stereocilia, but this is almost impossible, due to the necessity of solving Maxwell's equations for discrete electromagnetic modes within a complicated geometry (each stereocilium has a diameter of the order of the wavelength of light). Nevertheless, observations of the stereocilium structure⁴ enable one to make a rough estimate of the parameters involved in this fibre optic effect. The density of actin alone in the stereocilia is 0.4 g cm^{-3} . If we include the contribution of a cross-bridging protein, a reasonable estimate for the total protein density might be 0.55 g cm^{-3} in the stereocilium core. Using a specific refractive index increment of 0.18 for proteins, one finds a refractive index within the stereocilium of 1.43, as opposed to 1.33 for the surrounding endolymph (assumed to be that of water). Thus, the numerical aperture (NA) for this waveguide, which is a measure of the angular acceptance of the fibre, would be ~ 0.5 , a value quite similar to that which is obtained for the rod outer segments of the retina, a known biological fibre optic structure. The larger the NA, the greater the light-gathering properties of a fibre. This NA value might be compared with that of ~ 0.3 given by Kohlloffel for the body of the hair cell. Although the fibre characteristic term, R , for the stereocilium is quite small (1–3, as opposed to 10–20 for the hair-cell body) and the number of propagating modes will therefore be small for an isolated stereocilium⁵, it is quite probable that collective behaviour of the approximately 100 closely spaced stereocilia on each hair cell will be in the rule. Thus, the stereocilia bundle would still be observed macroscopically to behave as a light pipe.

This effect might be observed in several different ways. One would be to illuminate the cell externally, as Kohlloffel did, and look for localized brightness at the stereocilia tips. Alternatively, one might introduce a fluorophore which could freely diffuse within the cell. Light being selectively emitted from the stereocilia tips would then indicate the existence of the fibre optic effect described above. The latter approach already seems to have been tried for reasons other than verifying the existence of this effect. Goldstein⁶

diffused fluorescein diacetate (FDA), which is non-fluorescent and non-polar (lipid soluble), through the membrane of hair cells. The FDA is hydrolysed to fluorescein by nonspecific esterases within the cells. Fluorescein, which is fluorescent, is also polar, so it will not freely diffuse out through an intact membrane. A cell with a completely non-permeable membrane would then show fluorescence increasing with time as more and more FDA diffused in. Permeable cells would lose fluorescence as the rate of fluorescein diffusing out exceeded the rate of FDA diffusing in. The object of Goldstein's study was to determine changes in the permeability of hair-cell membranes as a function of sound exposure and oxygen deprivation. However, he observed a particular anomaly described as "spots of fluorescence at the top of each cell. . . . Because of the 'W' pattern of these dots, these presumably were the cilia of the hair cells". Because fluorescein does not bind to any known cellular structures, and because the light-piping effect was not considered, Goldstein assumed that the stereocilia are separated from the hair-cell cytoplasm by a unique membrane of their own. This postulated membrane would allow fluorescein to be retained within the stereocilia, causing the localized fluorescence. As no such membrane structure has ever been observed in detailed electron microscopic investigations, it is more probable that Goldstein's pictures are a good example of stereocilia light piping.

The importance of these points is underlined by the number of people using immunofluorescent localization of proteins in general within biological systems, and particularly those using this technique on hair cells. When one considers the striking resemblance of the photograph published by Macartney *et al.*¹ to those taken by both Kohlloffel³ (with external illumination) and Goldstein⁶ (with diffuse internal illumination), it is obvious that one cannot distinguish between the different sources of the illumination in these photographs. All light seems to be emerging from either the apical surface of the cells or the stereocilia in all three cases.

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BOOK REVIEWS

The order of life — towards a comparative biology

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WERNER Heisenberg once said that a revolution in science could be recognized by old terms acquiring new meanings and by an increase in philosophizing by its practitioners. Systematic biology is clearly in the midst of revolution and this is nowhere more clearly indicated than in these two splendid books. Their effect may be long in the making, however, for the shadow of Darwin has indeed fallen colossus-like over systematic biology for over a century and our concepts of biological order in space and in time are suffused with his ideas.

For Darwin, geographical distribution was an indispensable key to nature and he established what has become the traditional approach to this subject. He believed all the leading facts of geographical distribution to be explicable in terms of single centres of creation with subsequent migrations, largely due to chance dispersal, and further modification and multiplication of new forms. In the hands of his followers such as Matthew, Simpson, Darlington and Mayr, biogeography thus became concerned with centres of origin, means and routes of dispersal, climate and the evolution of dominance, and the analysis of biotic components in terms of colonizer elements. Despite the considerable achievements of these authors, their derogation being one of the least attractive aspects of both of these books, their biogeography can now be seen to be incomplete for the Herculean, often polemical, labours of Léon Croizat to combat these ideas are becoming increasingly well known. Arguing that the distributions of a wide variety of organisms over the face of the Earth were not random, but orderly, Croizat stressed *ad nauseam* that dispersal was no explanation. Recognizing that Earth and life evolve together, he pointed the way towards a synthesis of the geological and biological sciences.

For Croizat the history of life involves periods of mobilism, when species movement occurs in the absence of barriers to such movement, and periods of immobilism, when speciation occurs as the result of the erection of barriers and the fragmentation of ranges. This combination of geological-climatological and allopatric speciation events embraces the concept of vicariance. Thus we have a vicariance biogeography in which successive divisions of a parental biota replace the multiple and successive arrivals or sequential migrations of individual species. Sympatric distributions of closely

Vicariance Biogeography: A Critique. Edited by G. Nelson and D.E. Rosen. Pp.593. ISBN 0-231-04808-4. (Columbia University Press: 1981.) \$45.40. *Systematics and Biogeography: Cladistics and Vicariance.* By G. Nelson and N. Platnick. Pp.567. ISBN 0-231-04574-3. (Columbia University Press: 1981.) \$45.40.

related species are the primary evidence that dispersal has occurred.

Vicariance Biogeography contains the proceedings of a symposium held at the American Museum of Natural History in 1979, and on the evidence of the published version I am sorry that I missed it. Like most symposium volumes it is diverse, wide ranging and it contains articles covering the full spectrum from pro- to contravariance biogeography and its relationship to systematics. Brundin gives an especially valuable outline of Croizat's "panbiogeography" but it would be unfair to single out special praise in a volume of uniformly high quality which deals with almost all aspects of biogeography — vicariance, dispersal, speciation, plate tectonics, the testability of biogeographical theories and so on. An especially happy idea was that of allowing space for the discussions of each paper and then giving the author the right of reply, and frequently the more interesting points are made in these sections. The breadth and quality of this volume make it indispensable to biogeographers.

The relationship between systematics and biogeography is a recurring issue in both of these volumes. Croizat himself had little interest in the theory of systematics and it was only with the rise of phylogenetic systematics, now transformed to cladistics, that vicariance biogeography came into its own. The importance of phylogenetic analysis to biogeographical enquiry is now generally accepted.

The culmination, or *reductio ad absurdum* depending on one's viewpoint, of syntheses between cladistics and biogeography is provided by the book written by Nelson and Platnick. Both of these authors, individually and as a set, have written prolifically on the theory of systematics and biogeography, and it is therefore invaluable that they should have consolidated their views into a single testament. *Systematics and Biogeography* is divided into three unequal parts of uneven value — Form (systematics), Time (ontogeny, phylogeny, palaeontology) and Space (biogeography) — and is concerned with the theoretical structure of

comparative biology and the nature of our knowledge of organismic diversity. In a work laying much stress on methods, it is perhaps ironical that I find myself in agreement with its main conclusions while finding the philosophical and logical arguments leading to these conclusions naive.

In its treatment of biogeography we are taken beyond classical vicariance and the multiple sister-group approach of Hennig and of Brundin to a detailed analysis of organism and area cladograms, their comparison and congruity. The rigorous methods of comparing and summing cladograms, branching diagrams of relationship, yield a truly novel approach whose validity I would not question within the domain of its applicability, which concerns areas of endemism and their interrelationships. But species-level disjunctions, active or passive dispersal, extinction and non-allopatric speciation are all part of the history of life and cannot be legislated out of biogeography. Yet I look forward to the new rigour and energy that will surely be stimulated in comparative biology by this book.

Because of the importance of cladograms, the theoretical arguments behind the systematic sections of *Systematics and Biogeography* achieve a special significance. Here I find much with which to disagree. These sections are a detailed exposition of a particular or, some will say, narrow point of view. *Impunitum numquam beneficium* is the motto heading the book, but the authors need not fear that they will lack critical comment. *Quieta movere magna merces videbatur* may well prove to be their Sallustian refuge.

As a theoretical treatise the book is much concerned with the notions of order, existence and of relationship. The core of the approach, component analysis, involves the contention that cladograms are sets of phyletic trees only one of which is topologically equivalent to the cladogram, and it is cladograms, divorced of evolutionary implications, which have the greater generality and testability (propositions which I would deny) and which thus form the basis of systematic analysis.

Much of the early part of the book is concerned with the justification of cladograms as assessments of the order of nature, an order which presupposes the existence of some sets (taxa) and denial of others. But if we abandon the theoretical expectancy of a particular hierarchical order — that is, the evolutionary

background — then it must be acknowledged that in strict logic a system has all the orders of which it is capable, and that which is most familiar, or most immediately perceived, may not be *the* order. It was Kant's problem as to what justifies us in seeing nature as a whole that assumes the form of a logical system and its solution requires theoretical input concerning causality and not mere assessment of pattern. Cladistics as a formal system is a sterile enterprise; it becomes alive only when it deals in terms of generation and transformation, either as a result of human agency (for example the study of languages or of historical texts) or as a result of processes of nature (evolution). It follows that I find the authors' definitions of cladograms as synapomorphy schemes lacking such connotations less than useful, their explicit defence of this position notwithstanding. Others will decide for themselves.

There is also a great deal of set theory in the book, but of a simplistic and misleading sort. If one rejects nominalism, and accepts that sets over which quantification is possible exist, then one cannot arbitrarily deny some and not others. If quantification were not possible over all sorts of (admittedly overlapping) biological sets, then comparative physiology, ecology and so forth would not be possible. Logically, the meiofauna is as valid a set as is Aves. The special sense of existence for the hierarchical non-overlapping sets representing the higher taxa of biological systematics is meaningful only when causal principles of their origins are involved.

What can one make of discussions of sets that are "synonymous"? Does this mean identity, proper inclusion? This is not always made clear, and some set definitions given in terms of homologous character states may well prove to violate the "vicious circle principle". Concepts of intensional and extensional definition, denotation and connotation, although not explicitly discussed are often confused, and we could have hoped for more profound discussions of "natural" in the sense of "natural classifications", "natural taxa" and "natural kinds". The authors seem unaware that there is a vast philosophical literature dealing with such concepts and, it seems to me, fail to appreciate that these terms have meanings more precise than those of everyday use. We are told that homology does not exist independent of synapomorphy (a clear case of putting the cart before the horse!) any more than a set exists independent of its members, yet most logicians require the null or empty set. I am aware that logicians such as Goodman refer to wholes rather than sets, preferring to do without the null set, but there is no evidence that Nelson and Platnick have taken this particular route.

If I have dwelt on some of the logical and philosophical shortcomings, as I see them, of *Systematics and Biogeography* it is

because the relevant sections are a very important part of the work. And because they are so lucidly written, despite some lapses into condescension, these sections will have an immediate appeal to those biologists unused to logic and philosophy. Yet they must be read critically, for if it is indeed correct to accept the main conclusions of the book while denying the argument then the philosophy and logic must be re-worked. Nevertheless, I recognize the book as a *tour de force* of systematic biology; in particular, the synthetic parts such as those linking

systematics and biogeography into one grand structure are masterly. And even if in the historical sections scant attention has been made to my heroes, Kant and Goethe — whose notions of natural order are surely relevant to this book — I would still willingly sing with the angels from Faust: "Wer immer strebend sich bemüht, den können wir erlösen".

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Fresh interpretation of the galaxies

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A Revised Shapley-Ames Catalog of Bright Galaxies. By Allan Sandage and G.A. Tammann. Pp.157. ISBN 0-87279-652-3. (Carnegie Institution of Washington: 1981.) \$29.

SEVERAL years ago I walked into the refrigerated plate-storage room of the Dupont telescope at the Las Campanas Observatory in Chile, only to be confronted with a yellow wall of Kodak plate boxes carrying the message "Do not use — Reserved for Allan Sandage". These were the plates that were yet to be exposed to the black skies over the Chilean Andes, and which were destined to complete *A Revised Shapley-Ames Catalog of Bright Galaxies*.

Nearly half a century ago, Harlow Shapley and Adelaide Ames published their now-famous list of some 1,200 galaxies. Although this was only a small subset of the galaxies observed visually by the Herschels (data from which was compiled by Dreyer in the 1888 edition of the *New General Catalogue*), the *Shapley-Ames Catalog* bore the distinction of being quantified in a dimension that transcended the NGC and set the stage for subsequent discussions of the spatial distribution and intrinsic properties of galaxies. The original *Catalog* was, with a fair degree of success, a complete sample of the apparently brightest galaxies to a magnitude limit of about $m_{\text{B}} \sim 13$. The publication of a revised version by Sandage and Tammann is both a natural extension of this work and a bold expression of new convictions.

A Revised Shapley-Ames Catalog is not simply a listing of positions, magnitudes and classifications for the brightest galaxies, it is a personal statement about galaxies and the type of Universe they inhabit. The new *Catalog* contains much interpreted data, and in Sandage's own words "This Catalog is going to be controversial". Certainly, most of the controversy will arise from the distances that are hidden away in the tabulated

absolute magnitudes of Column 16. And almost as certainly we shall soon see a third edition of the *Reference Catalogue of Bright Galaxies* by the de Vaucouleurs, who, together with Corwin, aimed at providing a different interpretation of the data. The time and energy needed to follow the tortuous paths of logic encountered in determining the distances to galaxies can only leave lesser mortals following on in awe. So there is and will continue to be a widely accepted need for this type of quantitative speculation.

Still, for those wishing to strike out on their own the revised *Catalog* will provide a wealth of raw data. Radial velocities for all but a handful of galaxies are now available; a comprehensive reference list to the original data is graciously provided; and excellent photographs illustrate the classification system employed.

As dry as a catalogue might potentially be, there is a sprinkling of humour in the text. For example, in Table 10, describing the codes for the sources of plate material used in the galaxy typing, the Uppsala Schmidt is listed as having been used for a total of zero galaxies. One might wonder how many other telescopes should have also been included here.

Finally, it is of interest, to me in particular, that "at least 98 per cent of the catalog galaxies can be fitted into the revised Hubble system" while "descriptive terms, such as 'pec' (peculiar), 'disrupted', 'tidal', 'ring', 'jet' and 'edge on' have been added" only in several cases. Those who see the perfection and uniformity of nature give a balance to those who study the pathology.

As for controversy, this is surely a sign of health in an active science. *A Revised Shapley-Ames Catalog* will be a valuable and welcome addition to the continuing search for answers in extragalactic astronomy. □

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Epidemiology of the townsman's blight

D.G.T. Williams

Rural Conservation in Inter-War Britain. By John Sheail. Pp.272. ISBN 0-19-8232-36-5. (Oxford University Press: 1981.) £16.50, \$49.50. *The Countryside: Planning and Change.* By Mark Blacksell and Andrew W. Gilg. Pp.262. Hbk ISBN 0-04-711008-2; pbk ISBN 0-04-711009-0. (George Allen & Unwin: 1981.) Hbk £13, \$35; pbk £6.95, \$17.95.

A "widespread general concern" about the scale and nature of change in the countryside is (in the words of Blacksell and Gilg) "a relatively recent post-war development"; but, as Sheail argues, "the inter-war period was so critical and innovative that precedents can be found for almost every aspect of rural planning and conservation today". Together these two books offer a useful blend of historical perspective and identification of current problems, and they remind us that there is a rich vein of source material being tapped and yet to be tapped in studies of planning and the countryside in Britain. All of the authors are geographers: their approach, however, is widely based and of value to those concerned with economics, law, administration and other aspects of a complex subject. John Sheail, who is a historical geographer, has written his book as one of the Oxford Research Studies in Geography; Mark Blacksell and Andrew Gilg, both from Exeter University, have produced their volume as one of the Resource Management Series edited by Richard Munton and Judith Rees.

As a lawyer, I found that John Sheail's book offers a revealing analysis of the complicated and hesitant movement which culminated, after the Second World War, in comprehensive planning legislation dating from 1947. Against a background of the "economic and social malaise of the inter-war years" he examines a variety of experiments in local, regional and national planning secured through the initiative of legislators, officials and private pressure groups. In the country as a whole eloquent voices raised the spectre, in the words of Geoffrey Bournemouth (in one of the contributions to *Britain and the Beast* in 1938, a book which is mentioned by Sheail in his concluding chapter), of

the march of an inglorious suburbia across our countryside; the wanton sterilization of much of our most productive agricultural and market-garden land; the marring of vista upon vista, where country still remains, by the erection of unsuitable buildings, by thoughtless felling of trees, by Philistine methods of road-making and road-widening — in short the blighting touch of the townsman upon the country.

The countryside was seen as threatened as never before by the "suburbanizing trend" reflected in the spread of the towns and of townspeople, in greater mobility achieved through modern traffic and new

roads, in the search for new opportunities to enjoy the countryside for recreation or to adapt the countryside (allowing, for instance, for afforestation or new industrial growth) in the public interest.

One looks in vain for a consistent or positive approach in the inter-war years to the problem of reconciling the need for change and the desire to preserve. Rural conservationists succeeded in numerous battles but seemed a long way in 1939 from winning the war against what they saw as the evil of uncontrolled infringement of the old countryside. Sheail takes a balanced and understanding view of what they sought to achieve and how far they succeeded; and it is helpful to be told in the course of his book of the circumstances in which the Town and Country Planning Act 1932 was passed; of private legislation such as the Malvern Hills Act 1924 (later to be judicially considered, incidentally, by the House of Lords in *Pyx Granite Co. Ltd. v Ministry of Housing and Local Government* [1960] Appeal Cases 260); of the emergence of planning consultants and of the role of geographers in land-use surveys; of the work of such bodies as the Council for the Preservation of Rural England (founded in 1926), the Forestry Commission (established by statute in 1919) and the Youth Hostels Association (founded in 1930); of planning delays in the Ministry of Health and of "a somewhat ambivalent attitude" adopted towards statutory planning by the Ministry of Agriculture; and of numerous individuals (including Evelyn Sharp) who contributed in all sorts of ways to the national and local debates and controversies of the inter-war period. Drawing upon a wide range of original material, the author — admittedly on a selective basis, for the subject is vast — manages to develop a carefully structured account of the problems of planning and conservation.

The same problems are with us today, though the emphasis may have altered and the statutory framework has changed out of all recognition. In their work, Blacksell and Gilg set out to assess "the impact of the policies and plans that have proliferated during the past thirty years to guide the process of change in the rural landscape of Great Britain". They detect many inadequacies in the present system, notably the absence of co-ordinated guidance in the process of change. We are told about the work of the Ministry of Agriculture, Fisheries and Food, the Forestry Commission, the Department of the Environment, the Countryside Commission and the Nature Conservancy Council; and a number of complex issues are explored, often on a basis of original material (particularly drawn from the authors' own case studies in Devon), on such topics as the nature and measurement

of land use change, planning and land use, settlement planning, settlement change, and management in the countryside. For many readers a good deal of the authors' material will be unfamiliar, but there is clearly sufficient evidence of the value of the attempt by the Resource Management Series editors to provide "an interdisciplinary vehicle for major contributions from scholars and practitioners with a wide variety of academic backgrounds".

Problems of planning and conservation obviously loom large in a small and overcrowded country such as Britain — where the population, as Blacksell and Gilg point out, rose from 38.2 million in 1901 to an estimated 56 million in 1976. A study of the countryside alone becomes even more complex when one considers issues of pollution and the new and urgent demands for energy. Within their own terms of reference, these two books are important contributions to a subject of immense difficulty, riddled with conflicting interests, and they do much to correct what has perhaps been an overemphasis on town as opposed to country planning in the system which has evolved painfully over so many years. □

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Enshrouded by prose

David Davies

Cosmos and Creator. By Stanley L. Jaki. Pp.168. Pbk ISBN 0-7073-0285-4. (Scottish Academic Press: 1981.) £3.25.

PROFESSOR Jaki, a Benedictine priest with a long-time interest in the theological implications of science, has written *Cosmos and Creator* to make available the major points in his other books on creation "in a concise form for the wider public". In this I believe he flatters the wider public (in which I include myself) with a breadth of learning that it does not possess. A sentence such as

the presence of nominalism is merely nominal in the thought of Buridan and Oresme, while the presence there of a pre-Ockhamist traditional notion of creation is rather robust

is a typical example of some of the heavy weather encountered; I, for one, was grateful to my *Fontana Dictionary of Modern Thought* for providing the glossary that Jaki should have included. And even when all the words are easily understood, the author writes too often in an obscure way for my taste.

No less revealing should it seem that in Eddington's and Jeans' success of making a fad (very superficially, to be sure) [of] the inference

to God from science, the most effective part was played by a factor, apparently not at all mathematical. . . .

is a bit of a mouthful, particularly as the least of the many sins for which Jaki castigates Carl Sagan is for being a "consummate artist of sentences resting on double, triple and at times quadruple negatives".

Professor Jaki carries his argument through five chapters which read like five separate essays. In the first, "An Uneasy Fashion", he traces twentieth-century fashions in cosmology and the variable willingness amongst cosmologists to look through their specialization to the question of creation and a creator. His own view is that "he who says cosmos must say Creator in the traditional sense if man's sense of reality, purpose and consistency deserve more than lip-service", and he gives short shrift to some of those who have thought differently. Of course, proponents of the steady-state theory came into this category — continuous creation was "the most glaring trick ever given scientific veneer".

The next chapter begins with a text, or rather an anti-text from Anatole France — "The universe which science reveals to us is a dispiriting monotony. All the suns are drops of fire and all the planets drops of mud". Jaki rather ponderously rises to this bait by emphasizing the beauty of science, and then goes on to discuss whether the universe is necessary or merely contingent: the climate of thought in our time is not at all favourable for a recognition of reason's ability to bring within sight the contingency of the universe and its *raison d'être*, its having been created by a Being truly necessary.

Jaki then turns to look at creation from the viewpoint of Christian theology — not just as a vague general belief but as a dogma or proposition which demands unconditional assent. The *Fontana Dictionary* says that "dogma" is today "mostly used pejoratively, to mean an opinion held on grounds, and propagated by methods, which are unreasonable". I think that most scientists who wade their way through this difficult chapter, with its assertion that the first chapter of Genesis is the classic statement of the dogma of creation, will incline to the same view, and retreat to the position that religion is mainly an ethical matter and on many dogmatic matters they can at best be agnostic.

Following a chapter on the philosophical status of books, the relevance of which to the general thread of the volume escapes me even after four readings, Jaki concludes with a discussion on extraterrestrial intelligence — "this ultimate extension of Darwinism and an utterly self-defeating exercise in wishful thinking". Darwinism itself is for him a belief in the meaningless of existence and much to be reviled, although he does believe in evolution as an "imperfectly understood instrumentality of a species in the rise of another". Present-day advocates of taking ETI seriously look in it for a "final rebuttal of supernatural

revelation", and come in for some predictable stick. On the other hand, theists, for whom "intellects are a special creation of God", not a mere epiphenomenon of biochemical diversification, can keep an open mind about ETI and even look forward to a possible encounter with other intellects because both sides "will know something of a universal brotherhood based on a common dependence on the Creator".

I have read this book from a scientist's perspective, and maybe it looks different from a theologian's. But, much as it has

made me think, got under my skin and in some places stimulated me, I feel ultimately that it has helped me disappointingly little. The opacity, the dogmatism, the verbal tricks all speak to me of a failure by the author to reach out and understand his "wider public" and hold a helpful dialogue. Professor Jaki, it seems to me, wants to tell rather than to help. I think most of us need help. □

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A population problem for archaeologists

Colin Renfrew

Demographic Archaeology. By F.A. Hassan. Pp.298. ISBN 0-12-331350-3. (Academic: 1981.) \$32, £21.20.

FIFTEEN years ago, following the publication of Ester Boserup's stimulating work *The Conditions of Agricultural Growth* (Aldine, 1965), geographers and archaeologists fully realized that population, or population density, must no longer be regarded as a highly dependent variable, fixed or at least rigidly limited in Malthusian manner by environmental constraints. On the contrary, the mode of exploitation of resources could be varied according to need, and the level of population came to be seen rather as an independent variable (although not an unconstrained one) which would in part govern the way in which a society would intensify its agricultural production.

Since that time, demographic arguments, some of considerable sophistication, have loomed large in archaeological explanations. Their proponents have been encouraged by the new rigour of archaeological survey procedures, which have moved on from the casual serendipity of the weekend outing or the summer safari to the often exhausting demands of probabilistic sampling strategies and intensive field-walking by disciplined survey teams.

Demographic Archaeology undertakes a comprehensive review of demographic thinking and population models in contemporary archaeology. It ranges from the consideration of the population density of hunter-gatherer groups, where the context is very much one of biogeography, through the impact of sedentism and food production upon carrying capacity, and on to the emergence of complex, urban civilizations with their large population centres. As a general survey it succeeds admirably in bringing out the central role of demographic argument in much contemporary archaeological thought, and in summarizing the rather formidable range of quantitative formulations which have already been put forward.

One central weakness in the whole subject area, however, is that early population figures, for any given time and place, are extremely difficult to arrive at, based as they almost invariably are on fragmentary survey data, supplemented by the incomplete excavation of a few settlement sites. At present this often restricts an estimate of the population variable from rising beyond the purely notional.

The crucial question of estimating population size from archaeological data takes up only one 32-page chapter of Hassan's book. And while this chapter is a very competent review of what has been written, it is not a very critical one, nor does it, to my mind, bring out the still unresolved difficulties in estimating population figures with any degree of accuracy and reliability from archaeological data. Until these difficulties are overcome, the demographic explanation in archaeology must remain something of a will o' the wisp: an enticing hypothesis, the testing of which remains a frustrating task. Hassan might have dealt at greater length with this crucial problem, for it is in the improved estimation of early population figures that the future of demographic archaeology must lie, rather than in the production of ingenious theories which, however attractive and plausible, have merely the status of speculation until sustained by acceptable data from the field.

Despite this central difficulty, which is not Hassan's problem alone but one which faces all researchers, his book is undoubtedly a sustained and coherent contribution to archaeological theory. It serves to bring together a whole series of ideas never before so effectively related, and takes its place at once among the small number of books on archaeological theory which rise above mere polemic to serve as valued works of reference. □

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